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(54) Title: AGENTS INTERFERING WITH THE BINDING OF PROTEIN TYROSINE PHOSPHATASE PEST TO DOMAINS OF SIGNALLING PROTEINS AS INHIBITORS OF CELL MIGRATION AND/OR OF FOCAL ADHESION			
(57) Abstract <p>This invention relates to agents or compounds capable of interfering with the binding of protein tyrosine phosphatase PEST to protein domains of signalling molecules involved in cell migration, focal adhesion and/or cell proliferation, namely p130cas and paxillin. The agents can be derived from the minimal sequences found in binding studies. PTP-PEST is a conserved phosphatase essential for embryo development. Knock-out cells (PTP-PEST -/-) have been perpetuated from null embryos and they show defects in cell migration, focal adhesion and cell proliferation. Therefore, any agent capable of interfering with the activity of PEST in a diseased target tissue, is considered to be a potential therapeutic agent to treat any disease having any of the following etiological components: cell proliferation, cancer, metastasis, inflammation, and angiogenesis. This invention further relates to a method for finding genuine substrates for enzymes, namely phosphatases, combining gene targeting knock-out technique and substrate-trapping technique with the aid of a substrate binding inactive mutant enzyme. By using a gene targeting knock-out technique, there are less artefacts than by using other techniques (using vanadate compounds, for example) wherein an artificial non-specific increase of the level of hyperphosphorylation occurs.</p>			

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TITLE OF THE INVENTION

Agents interfering with the binding of protein tyrosine phosphatase PEST to domains of signalling proteins as inhibitors of cell migration and/or of focal adhesion

5 FIELD OF THE INVENTION

The invention relates to agents modulating focal adhesion and cell migration, particularly those modulating the phosphorylation on protein tyrosine residues.

BACKGROUND OF THE INVENTION

10 Phosphorylation on tyrosine residues is an important mechanism for transmitting extracellular stimuli in biochemical and cellular events such as cell attachment, mitogenesis, differentiation and migration (for review see (1)). Protein tyrosine phosphorylation levels are regulated by the function of two protein families: the protein tyrosine kinases (PTK) and the protein tyrosine phosphatases (PTP). All
15 PTPases have a conserved catalytic domain characterized by the signature motif [I/V]HCxAGxxR[S/T]G. Biochemical and kinetic studies demonstrated that the cysteine residue found in this signature motif is essential for catalytic activity of PTPs since mutation of this cysteine completely abolishes PTPase activity (2).

20 PTP-PEST is a stable soluble PTP that is ubiquitously expressed throughout embryonic development and in murine adult tissues (3). The N-terminal portion of the enzyme encodes for the catalytic domain while the C-terminus portion is composed of five proline rich domains (4) and a binding site for the adaptor protein Shc (5). Among the proline rich domains, two proline rich motifs are recognized by the SH3 domain of other signalling molecules. The Pro4 (PPLPER) motif was shown to promote
25 interaction with the SH3 domain of the csk kinase. This same pro4 motif in conjunction with the pro1 (PPKPPR) motif interact with the SH3 domains of the GRB2 adaptor protein. This interaction was proposed to facilitate the recruitment of PTP-PEST to an activated epidermal growth factor receptor in order to dephosphorylate a substrate of approximately 120Kd (6). Indeed PTP-PEST Pro 1 domain was found recognized to
30 play a major role in the association of PTP-PEST with its only known substrate to date, the adaptor protein p130cas (crk associated substrate) (6). Using PTP-PEST deficient fibroblast, we recently shown that, indeed in the absence of its PTPase regulator, p130cas becomes constitutively phosphorylated on tyrosine (7), indicating the physiological relevance of the pro1 of PTP-PEST to the SH3 domain of p130cas.

35 Recognized as a direct target for the YopH PTPase during yersinia infection (8), p130cas was identified as a major substrate for tyrosine kinase in v-crk transformed cells (9). In normal cells, p130cas become highly phosphorylated following integrin dependent activation of the fak and src kinases (10, 11). This phosphorylation appears to allow a series of tyrosine dependent signalling that has among other consequences

the actin filament reorganization. Because of the importance of integrin signalling in cell cytoskeleton, motility and transformation, the action of PTP-PEST on p130cas may have dramatic consequences in mammalian development as well as in some physio-pathological events. The process of cell migration is crucial for the correct development 5 of a mammalian embryo. In an adult organism, cell migration still plays an important role in events like invasion of a wounded space by fibroblasts and endothelial cells and translocation of lymphocytes and neutrophils to an inflammation site. In cancer, tumor 10 cells also have to migrate in order to reach the circulatory system and disperse throughout the organism. It is therefore important to assess the physiological importance of PTP-PEST and of substrates thereof, such as p130cas in cell motility 15 by providing PTP-PEST negative cells. Inhibitors of this enzyme could be valuable therapeutic agents.

The driving force for cell mobility is actin polymerization/depolymerization, which 20 is under the control the Rho family of G proteins. The three most studied members of this family, rho, rac and cdc42, control respectively the formation of focal adhesions, membrane ruffles and filopodia. Membrane ruffles and filopeida consist of actin-rich membrane protrusions that a cell uses to extend itself forward; the difference between the two lies in the fact that the ruffles are formed by a network of intertwined actin 25 filaments that shape them, whereas filopodia assume a hair-like conformation.

Focal adhesions are the sites of contact between the extracellular matrix and 30 the cytoskeleton through the integrin family of transmembrane proteins (12). They contain many structural proteins such as talin, tensin and α -actinin, as well as important tyrosine kinases like FAK, src, and csk, that are activated upon extracellular matrix binding. Multimeric protein complexes are then formed which contain many different adapter proteins, such as p130cas, shc, grb2, crk and nck. These interactions confer an important role to focal adhesions in signal transduction pathways. They also represent the most important site of tyrosine phosphorylation in a cell, to a point where, following plating of cells on an extracellular matrix, focal adhesions can be stained 35 using anti-phosphotyrosine antibodies.

If the identification of proteins involved in focal adhesion formation and 40 disassembly proceeds at a rapid rate, the roles that each of them play only slowly start to be elucidated. Recent results show a direct correlation between FAK activity and migration speed, and that this effect was the result of a hyperphosphorylation of p130cas by the src kinase (13). The viral form of src, v-src, also increases focal adhesion turnover in the transformed cell, and its catalytic activity was shown to be 45 required for this effect (14).

Much less is known about the roles of protein tyrosine phosphatases (PTP) in general, and in cell migration in particular. One exception is the bacterial PTP Yersinia 50 YopH that, once translocated in a cell, dephosphorylates p130cas and FAK, and

destabilizes focal adhesions (8). It appears that p130cas has an important role to play in focal adhesion.

We studied the role of a PTP that has p130cas as its main substrate, PTP-PEST (6). PTP-PEST contains the typical phosphatase catalytic domain and several proline-rich regions that were shown to interact with several signalling proteins like p130cas, Grb2 (4) and paxillin (15). Furthermore, we also reported that a NPLH motif was responsible for a constitutive association between PTP-PEST and the PTB domain of the adaptor protein Shc (5).

Even though PTP-PEST, like the other members of its family, was described as a cytosolic protein (3), we have recently shown that it translocates to the membrane periphery after cell attachment to fibronectin.

Identification of physiological substrates of protein tyrosine phosphatases is a key step in understanding the function of these enzymes. Mutations of invariant amino acids in the catalytic domain of PTPases produce inactive enzymes that are nevertheless able to complex with their tyrosine phosphorylated substrates.

As a mean to isolate putative substrate of PTPs, cysteine 215 to serine or aspartic acid 181 to alanine mutants within the catalytic domain of PTP1B have been identified as substrate trapping intermediates (2). Such mutants are an important tool in the study of the functions of PTPs. Unfortunately, before performing substrate trapping experiments, the phosphotyrosine content of the protein source must be increased. Two approaches are currently used: 1- physiological stimulation of cells, such as with EGF, triggers a cascade of tyrosine phosphorylation events (4) but will result in the tyrosine phosphorylation of a limited number of proteins and 2-treatment of cells with pervanadate of inhibit intracellular PTPases (6) will result in a very high number of proteins being tyrosine phosphorylated, but some of them not on physiological sites giving rise to potential artifacts. Overall, it is unlikely that both of these approaches will result in the identification of all genuine substrates of a given PTPase and alternative strategies should be envisaged.

In view of the foregoing, there is a need to investigate the physiological importance of phosphotyrosine phosphatases and their substrates. There is further a need to elucidate the role of PTP-PEST in cell migration and to provide agents capable of interfering therewith, and this, for the purpose of developing therapeutic agents.

SUMMARY OF THE INVENTION

This invention provides novel therapeutic agents for treating diseases having any of the following etiological components: cell proliferation, cell migration, inflammation and angiogenesis.

Such novel therapeutic agents are derived from the entities participating in the complexing of the protein tyrosine phosphate PEST (PTP-PEST) with signalling

molecules involved in cell migration, focal adhesion and cell proliferation.

In a specific embodiment, the signalling molecules are p130cas and paxillin, the former being the only substrate known up to date for PTP-PEST.

Binding studies involving these two substrates resulted in the finding of peptides retaining binding capacity to the substrates. These peptides are prototypes of agents capable of interfering with the binding of PTP-PEST to its substrate(s). These peptides are considered to be therapeutic agents capable of competing with the native PTP-PEST for the binding of substrates or other signalling molecules.

Another object of the invention is the finding of genuine substrates of enzymes such as phosphatases. Fibroblasts rendered null for the expression of PTP-PEST have a set of tyrosine hyperphosphorylated proteins. This hyperphosphorylation state is detectable by the use of ligands such as anti-phosphotyrosine antibodies. These null cells have been used in combination with a substrate-trapping mutant enzyme, which catalytic site has been rendered inactive. As a result amongst the hyperphosphorylated proteins, p130cas has been identified as the sole substrate for PTP-PEST in fibroblasts.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS OF THE INVENTION

This invention will be described hereinbelow by referring to specific examples and appended figures, which purpose is to illustrate the invention rather than to limit its scope.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: 1a, b and c illustrate the making of a PEST null mouse.

Figure 1a) is a targeting vector map of the vector used to create a PTP-PEST null mouse;

Figure 1b) is a Southern blot of ES cells showing a 2 KB deletion in PEST allele; and

Figure 1c) is a Western blot of embryos of different genotypes (+/+, +/- and -/-).

Figures 2.1 to 2.8 show the early evolution of the deformities observed in PTP-PEST null mouse embryos.

Figure 3 shows a Western blotting of -/- and wt embryos using anti-phosphotyrosine antibodies.

Figure 4 Gene targeting of the PTP-PEST suppresses fibroblast motility on the extracellular matrix fibronectin. Monolayers of each cell line were wounded and maintained at 37°C for 72 hours before fixing. The ability to migrate into the wound was monitored by phase contrast microscopy of unstained cells which were photographed (100 X magnification). The aspect of each wound represents the typical result obtained after 5 independent experiments. Migration is affected in panel B(-/-) compared to panel A (+/-).

- Figure 5** Immunofluorescence images of PTP-PEST heterozygote (a,b,e,f) and homozygote (c,d,g,h) cells plated on fibronectin. After 25 minutes (a-d), there are no qualitative differences on the actin filaments, stained using a rhodamine-phalloidin conjugate (a,c) or in the focal adhesions, stained with a anti-vinculin antibody and highlighted using a FITC-conjugated second antibody (b,d). However, when the cells were left for 3 hours before fixing, the +/- cells became rounded (e) and only formed punctual focal adhesions at their periphery (f), whereas the -/- cells continued to spread, forming numerous stress fibers (g) and large focal adhesion plaques scattered throughout their ventral surface (h). Magnification: 1000 X.
- 5 **Figure 6** Constitutive tyrosine phosphorylation in PTP-PEST (+/-) and (-/-) cells of cortactin, FAK and paxillin. p130cas was already shown to be a substrate for PTP-PEST (6) and to be hyperphosphorylated in the PEST(-/-) cells and is included here as a control. Only paxillin was found to be hyperphosphorylated, and this phosphorylated form corresponded to the upper band of the paxillin doublet obtained 10 when blotted with the anti-paxillin antibody (right panel). Tyrosine phosphorylation of the focal adhesion component vinculin could not be detected in either cell line, possibly 15 because the cells were not stimulated.
- 10 **Figure 7** Constitutive phosphorylation of PSTPIP in unsynchronized PTP-PEST(+/-) and (-/-) cells. PSTPIP was immunoprecipitated in non-denaturing conditions and 20 probed with a HRP-conjugated anti-phosphotyrosine antibody. PSTPIP was hyperphosphorylated in the (-/-) cells and seems to form more complexes with other, yet-unidentified tyrosinephosphorylated proteins (right lane).
- 15 **Figure 8** Staining of unsynchronized PTP-PEST (-/-) cells plated on uncoated tissue culture glass slides using rhodamine-conjugated phalloidin. The arrows indicate the cleavage furrows of two pair of cells found in M-phase inside the same field. Magnification: 400 X.
- 20 **Figure 9** Southern blot and Northern blot analyses of DNA and RNA isolated from the PTP-PEST+t- and -/- cell lines established from primary cultures of embryos isolated from the PTP-PEST knock-out mice. A) Genomic DNA was digested with BamHI and 25 probed with a KpnI/SacI fragment of the PTP-PEST cDNA. The 12 kb band corresponds to the wt allele while the 7 kb band corresponds to the targeted allele. WT DNA was also included as a positive control. B) a transcript of 3.8 kb was detected in PTP-PEST +/- cell lines but was absent in the PTP-PEST-/- cell lines by Northern blotting. RNA isolated from WT fibroblasts was included as a positive control (upper 30 panel). The blot was also probed with GAPDH (lower panel) to ensure equal loading.
- 35 **Figure 10** Analysis of the phosphotyrosine profile of the PTP-PEST +/- and -/- cell lines. A) 15 pg of lysate of PTP-PEST +/- and -/- was analysed by antiphosphotyrosine immunoblotting (first two lane). Antiphosphotyrosine immunoprecipitations from the PTP-PEST +/- and -/- cell lines were also analysed by antiphosphotyrosine

immunoblotting. Hyperphosphorylated proteins of 180, 130 and 97 are detected in the PTP-PEST-/- cells. B) p130cas is hyperphosphorylated in the PTP-PEST deficient cell line (PTP-PEST-/-). Upper panel, p130cas was immunoprecipitated from PTP-PEST +/- and -/- cell lines and the phosphorylation level was analyzed by 5 antiphosphotyrosine immunoblotting using the 4G10 monoclonal antibody. Lower panel, equal amounts of p130cas in the TCL and immunoprecipitates was confirmed by stripping the blot and reprobing with anti-p130cas B+F.

Figure 11 Substrate trapping experiment in PTP-PEST deficient cells (-/-) lysate denotes p130cas as a physiological and specific substrate for PTP-PEST. The 10 substrate trapping experiments were performed by incubating 1 mg of cell lysate from PTP-PEST +/-, -/- and pervanadate treated COS-1 cells with 100 ng of either GST-PTP-PEST WT (aa 1-453) or GST-PTP-PEST C231S (1-453). The bound 15 proteins were analyzed by antiphosphotyrosine western blotting using 4G10 monoclonal antibody (top panel) or, after stripping the blot, with anti-p130cas B+F (middle panel). A coomassie blue stained gel of the GST fusion proteins used in the substrate trapping is shown in the bottom panel to show integrity of products and as a loading control. Two arrows are drawn next to the pp130 (phosphorylated p130 proteins) to emphasize the diffuse band.

Figure 12 PTP-PEST associates with the SH3 domains of p130cas, Hefl and Sin *in* 20 *vitro*. HA-PTP-PEST transfected COS-1 cell lysate (1 mg) were incubated with 100 ng of either the GST linked SH3 domains of p130cas, Hefl, Sin or with GST alone prebound to glutathione sepharose. Following extensive washing, the proteins were eluted, separated by SDS-PAGE and analyzed by western blotting for the presence 25 of HA-PTP-PEST using the 12CA5 monoclonal antibody (left panel). In the right panel, a coomassie blue stained gel of the GST fusion proteins used in the *in vitro* binding assay is shown to verify integrity of the products.

Figure 13 PTP-PEST proline rich region 1 (Prol) is responsible for the interaction with 30 the SH3 domains of the family of adaptor molecule p130cas, Sin and Hefl. A) Schematic representation of the PTP-PEST GST fusion proteins encoding for the different proline rich region of PTP-PEST (Prol-5) used in the farwestern binding assay, B) Coomassie blue stained gel of 100 ng of the PTP-PEST fusion proteins used in the farwestern assay and is also representative of the protein found on the PVDF membrane. Three blots containing the PTP-PEST fusion proteins were respectively 35 incubated overnight with [³²P]radiolabeled GST SH3 domains of p130cas (C), Sin (D) and Hefl (E) and following extensive washing, the blots were exposed 20 min to X-ray film.

Figure 14 PTP-PEST and p130cas associate *in vivo*. Myc-tagged p130cas vector or a Myc SH3 domain p130cas vector was co-transfected with HA-PTP-PEST, WT or C231S, in 293T cells and the presence of the various Myc tagged p130cas proteins

were analyzed in the 1075 immunoprecipitates of the PTP-PEST protein. A) schematic representation of the myc-tagged p130cas proteins used in the coimmunoprecipitation experiment and B) Following coimmunoprecipitation and extensive washing of the complexes, the bound proteins were resolved on SDS-PAGE, transferred to PVDF and analyzed by western blotting using anti-Myc 9E10 monoclonal for the presence of the various Myc tagged p130cas proteins. As a negative control, HA-tagged T-cell PTP (HA TC-PTP) was cotransfected with the Myc p130cas construct and was immunoprecipitated with anti-HA 12CA5. The full length Myc-p130cas proteins migrate just above 130 kDa and the MycSH3 domain of p130cas migrates at 30 kDa. C) Western blotting of the TCL with 9E10 and 12CA5 antibodies to visualize the different Myc-p130cas proteins, the HA-PTP-PEST and HA-TC-PTP respectively.

Figure 15 Paxillin is associated with PTP-PEST in various mouse tissues. PTP-PEST was immunoprecipitated from 1 mg of liver, brain, heart, kidney, lung, spleen and thymus lysates and the presence of associated paxillin f was ascertained by western blotting using an antibody against paxillin. Paxillin was found in PTP-PEST immunoprecipitations from liver, brain, heart, lung, spleen and thymus but not in kidney lysates. No paxillin was detected in preimmune immunoprecipitation from liver lysate.

Figure 16 Pro 2 of PTP-PEST is required for paxillin binding in NIH 3T3 cells. A) Schematic representation of the PTP-PEST GST fusion proteins used in the binding assays. Deletion mutants (B) or proline rich motifs (C) of the PTP-PEST C-terminus were incubated with NIH 3T3 cell lysate and bound paxillin was monitored by western blotting (top panels). A Coomassie blue stained gel of the fusion proteins used in the binding assay is shown in the bottom panels of B and C.

Figure 17 Pro 2 of PTP-PEST is essential for binding to paxillin in vitro and in vivo. A) HEK 293-T cells were transiently transfected with either HA-WT or HA ΔPro 2 PTP-PEST. 200 µg of the indicated lysates were incubated with either GST Paxillin N, GST Paxillin C, GST SH3 p130Cas or GST alone. Bound PTP-PEST was monitored by western blotting with the anti-HA antibody 12CA5 (Top panel). 5 µg of TCL were also blotted with the 12CA5 antibody to verify comparable expression of HA-WT and HA-ΔPro 2 PTP-PEST. The presence and integrity of the GST fusion proteins was verified by reprobing the blots with a polyclonal antibody against GST. B) HEK 293T cells were transiently transfected with either: Mock (empty pACTAG), HA-WT, HA-ΔPro1 or HA-ΔPro 2 PTP-PEST plasmids. The proper expression of each construct was verified by immunoblotting 5 µg of TCL with the anti HA antibody 12CA5 (Top panel). Paxillin was immunoprecipitated with a monoclonal antibody and the presence of PTP-PEST was assessed by immunoblotting with the anti-HA antibody 12CA5 (Middle panel). Equal precipitation of paxillin from each sample was verified by reprobing the blot with a monoclonal antibody against paxillin (Bottom panel).

Figure 18 Mutant P362A of Pro 2 on PTP-PEST abolishes binding to paxillin in vitro.

A) Schematic representation of the proline to alanine mutants of the GST PTP-PEST Pro 2 construct (aa 344-437). The region shown represents the paxillin binding site identified in figure 3. B) The GST Pro 2 as well as the eight proline to alanine mutants were purified and incubated with 200 µg of NIH 3T3 cell lysate. Bound paxillin was monitored by immunoblotting with a monoclonal antibody against paxillin. C) To verify expression as well as integrity of the fusion proteins used in the binding assay, an aliquot of each binding assay was separated by SDS-PAGE and the gel was stained with coomassie blue.

5 Figure 19 LIM domains 3 and 4 of paxillin are required for binding to PTP-PEST in vitro. A) Schematic representation of a panel of paxillin LIM domains GST fusion proteins used to identify the PTP-PEST binding site. B) The paxillin GST fusion proteins were purified and incubated with HEK 293T cell lysates transiently expressing HA-PTP-PEST. Bound PTP-PEST was detected by immunoblotting with the anti-HA antibody 12CA5 (top panel). The expression as well as the integrity of each fusion protein used in the binding assay was verified by Coomassie blue staining of a gel containing the resolved proteins.

10 Figure 20 LIM domains 3 and 4 of paxillin are required for binding to PTP-PEST in vivo. HEK 293-T cells were transiently co-transfected with HA-PTP-PEST and either pcDNA3, WT, ΔLIM3 or ΔLIM4 avian paxillin. A) 48 h after transfection, paxillin was immunoprecipitated from each lysates with a avian specific polyclonal antibody and the co-precipitation of PTP-PEST was assayed by immunoblotting with the anti-HA antibody 12CA5. B) The blot was reprobed with a monoclonal antibody against paxillin to verify equal precipitation. No paxillin was detected in the empty vector (pcDNA3) sample. C) HA-PTP-PEST expression levels were monitored from each transfection by immunoblotting 5 µg of TCL with the anti HA antibody 12CA5.

15 Figure 21 Intact LIM 3 and LIM 4 of paxillin are required for binding to PTP-PEST. pcDNA3, WT or mutant paxillin (ΔLIM 3, C467A, C470A, C467/470A, ΔLIM4 and C523S) were in vitro translated in the presence of ³⁵S-methionine. These in vitro translated products were incubated with GST-PTP-PEST Pro 2 (344-397). Following 20 repeated washes of the GST matrix, bound proteins were separated on 10% SDS-PAGE and bound proteins were visualized by a 8 hour exposure to film (top panel). GST alone was incubated with WT paxillin and serves as a negative control. In the bottom panel, 15% of the amount of each paxillin products used in the binding assay demonstrate integrity of the products (8 hours exposure).

25 Figure 22 Paxillin is not a substrate for PTP-PEST. Lysates of NIH 3T3, NIH 3T3 expressing Src Y527F and NIH 3T3 treated with pervanadate were incubated with four different PTP-PEST GST fusion proteins: C-Terminus (aa 276-775), 1-453 WT, 1-453 C231 S and 1-354 C231 S. A) Bound proteins were resolved by SDS-PAGE and tyrosine phosphorylated proteins were detected by immunoblotting with an

antiphosphotyrosine antibody. The blot was reprobed with anti paxillin (B) and anti-p130Cas (C) monoclonal antibodies. D) The expression and integrity of each fusion proteins was verified by Coomassie blue staining of the PVDF blots.

Figure 23 Model for PTP-PEST functions in the disassembly of focal adhesions. A)

- 5 Simplified model of the proteins involved in focal adhesions. PTP-PEST is recruited to focal adhesions by a yet unknown mechanism (14). It can then bind to the LIM 3-4 of paxillin and subsequently to the SH3 domain of p130Cas and dephosphorylate tyrosine residues (pY) on p130Cas essential for binding SH2 domain containing protein (such as Crk). By dephosphorylating p130Cas, PTP-PEST is believed to inhibit downstream signalling from Src, SOS and C3G. Csk associated to PTP-PEST can also inhibit Src by phosphorylating the negative regulatory sites of Src. B) The consequence of PTP-PEST recruitment to focal adhesions is the binding to paxillin and p130Cas. Both the LIM 3 of paxillin (19) and the SH3 domain of p130Cas (40) are essential for targeting these proteins to focal adhesion. By binding to PTP-PEST, we propose that they both of these proteins would relocate to the cytoplasm. Focal adhesion formation is further inhibited by the inactivation of Src activity by PTP-PEST associated Csk. These events implicating PTP-PEST would favor the breakdown of focal adhesions and enable cell migration before focal adhesions are reassembled.

- 10 **Figure 24** illustrates alignment the complete sequences of mouse and human PEST (SEQ ID NOs: 1 and 2, respectively). The proline rich domains are boxed and are very conserved amongst species.

15 Using the fibroblast cell lines where PTP-PEST was removed by gene targeting, we studied the migration capabilities and the number of focal adhesions in cells lines homozygote and heterozygote for the mutation. Since the (-/-) cells showed a marked decrease in migration and increase in focal adhesions, we then verified the phosphorylation state of important tyrosine phosphorylated proteins found in focal adhesions. The one that was found to be constitutively hyperphosphorylated, besides p130cas which was already shown to be a substrate, was paxillin. Paxillin was recently shown to be associated with PEST (15) but it is not known if it is a physiological substrate for this phosphatase. From this information, we concluded that PTP PEST plays an important role in cell migration, possibly by helping the breakdown of focal adhesions via the dephosphorylation of p130cas and/or paxillin, directly or indirectly.

20 During our experiments with the PEST (-/-) cells, we now observe a high occurrence of cells with two nuclei separated by a cleavage furrow, a phenotype we first associated with the migration impairment that would slow the process of cell division and increase the population of cells found in M phase. A cleavage furrow-associated protein that is also a substrate for another PEST-like phosphatase, PTP HSCF, was recently cloned (16). Since this protein, called PSTPIP, associates with a domain on PTP HSCF that is also found in PTP PEST, we verified the phosphorylation

state of PSTPIP in the (-/-) cells to find that it was also hyperphosphorylated. So, whether PSTPIP is a direct substrate or not, we concluded that PTP PEST could also play a role in cytoskeleton rearrangement during cell mitosis. This reinforces our assumption that interfering with the binding of PTP-PEST with its substrate(s) would provide valuable therapeutic tools (anti-inflammatory, anti-angiogenic and/or anti-tumor agents).

EXAMPLE 1

Construction of a knock out mouse PTP-PEST (-/-):

A targeting vector was generated following the genomic mapping of the PTP-PEST locus, that eliminate exon 4 to exon 7 of PTP-PEST gene encoding essential domain of the PTPase catalytic domain. Following electroporation of 2×10^7 J1 embryonic stem cells (13,14) 7 out of 223 clones lost one of the wt allele and presented the 7.0 kb diagnostic band by Southern blot analysis. Chimaeric mice were generated with two independently isolated +/- cell line and germline transmission of the targeted allele was obtained with both. Crossing heterozygous mutants resulted into a 2 to 1 ratio of heterozygotes to wild type animals and the complete absence of homozygous animals, demonstrating that absence of PTP-PEST resulted in embryonic lethality. The heterozygotes animals remain asymptomatic throughout their normal lifespan. Dissection of pregnant heterozygous mothers show that homozygous mutants appear abnormal by 9.5 embryonic day (9.5 dpc). In most homozygous embryos, visible examination show a clear retardation of caudal development, a failure of neural tube closure and of embryo turning by day 9.5. However, few embryos reach 10.5 dpc of development, but show a significant reduction in size.

Histological analysis of various embryos indicate that at 7.5 dpc normal gastrulation appear to be present with the three germ layers appearing morphologically normal (Fig 3.1). Similarly, typical yolk sack formation is present in all embryos detected (Fig 3.2 b). The first indication of abnormal development is seen at 8 dpc where the neuroepithelium development seems to be defective (data not shown). Examination of 9.5 dpc homozygous embryos led to the detection of clear defect during the late neural tube development and somite formation (Fig. 3.3 to 3.5). These defects are more striking in the caudal region. Finally by 10.5 dpc degeneration of mesenchyme and neurepithelium has progressed significantly on those that survived and went through embryo folding. Strikingly, the phenotype is seen as a deformed embryo with receding caudal region (Fig. 3.6). This is confirmed by the histological section of the embryo. In some homozygous embryos, a striking mesenchymal manifestation seen is the depletion/absence of splanchnopleure in the poorly developed gut epithelium (Fig. 3.6). In the mutant embryo, the mesenchyme comprising the septum transversum is depleted and in degeneration (Fig. 3.7). The hepatic diverticulum fails to develop and no fetal liver develops (Fig. 3.8).

Because of this last observation and the known PTP-PEST expression in the developing and adult liver we investigated specifically the hepatic developing system. As a first step, we looked for the presence of diagnostic hepatocyte mRNA by *in situ* hybridization. We focused on the albumin mRNA expression, because it is expressed at the onset of hepatic specification and is restricted to the developing hepatocytes (41). We could detect albumin mRNA as early as 9.5 dpc in wild type embryos no albumin expression could be found in the PTP-PEST null embryos indicating that the hepatic development was perturbed at the earliest stages of development. This was also the case for the 10 dcp embryos as which stage, in normal embryos, the liver is one of the most proeminent organs (data not shown). We also analyzed HNF-3a expression which is normally found in the developing liver, gut, notochord and floorplate of the neural tube (42-45). As with albumin, HNF-3a was not detected in any PTP-PEST -/- cells which resembled hepatocytes. However, gut, notochord and the floorplate of the neural tube all stained positive with HNF-3a message indication that the development of tissues, at least at a superficial level was relatively unaffected by the absence of PTP-PEST.

The possibility remain that some of the defect could be caused by indirect effects on vascularization. Although the early vascular system appear normal, in order to verify more precisely its status in the homozygous embryos, heterozygous animals were mated with a transgenic line expressing the lacZ reporter gene under the transcriptional control of the endothelial-specific tek promoter. Heterozygous PTP-PEST embryos carrying the transgene were backcrossed to obtain homozygous PTP-PEST embryos carrying the transgene. Homozygous-lacZ-tek carrying PTP-PEST E9 mutants were stained with Xgal and whole mount and cross-section of these embryos were examined. Homozygous animal possessed a normal but slightly retarded developing vasculature, confirming that vascularization was not responsible for the unique phenotypic appearance of the -/- PTP-PEST embryos.

The appearance of hyperphosphorylated p130cas in the -/- fibroblasts was an important indicator of the physiological relevance of the PTP-PEST p130cas interaction. In order to verify if p130cas as well as other potential substrates could be detected, wt and -/- embryos were isolated, directly lysed into SDS-PAGE lysis buffer, and subjected to Western blot analysis using a antiphosphotyrosine antibody. A major band of 130 kd was hyperphosphorylated.

EXAMPLE 2:
35 **CELL MIGRATION AND FOCAL ADHESION ARE AFFECTED IN PTP-PEST (-/-) CELLS:**

Materials and Methods

Antibodies

The mouse polyclonal anti-PIP antibody raised in our laboratory against PIP C-

terminus is described in (75). The mouse monoclonal anti-cortactin is a gift from Dr. J. Thomas Parsons, University of Virginia, Charlottesville, VA. The following antibodies were purchased as indicated: rabbit anti-FAK (A17) and rabbit anti-p130cas (C20) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); mouse anti-paxillin from Transduction Laboratories (Lexington, KY); mouse anti-vinculin (hVIN-1) from Sigma (St-Louis, MO); horseradish peroxidase (HRP)-conjugated anti-mouse and -rabbit IgG antibodies as well as FITC-conjugated anti-mouse antibody from Jackson Immunoresearch laboratories, Inc. (West Grove, PA). The anti-phosphotyrosine antibodies used were a mouse HRP-conjugated anti-phosphotyrosine antibody (pY20) from Transduction Laboratories and the mouse monoclonal 4G10.

10 *Cell culture*

The PTP-PEST +/- and -/- cell lines have been previously described. The cells were maintained in DMEM medium supplemented with 10% serum, L-Glutamine and Penicillin/Streptomycin .

15 *Wound-healing migration assays*

The capacity of each cell line to migrate on fibronectin was monitored as follows: Falcon chamber slides (Becton Dickinson, Franklin Lakes, NJ) were coated overnight at 4°C with a solution of fibronectin (10 µg/ml) in phosphate-buffered saline (PBS, 10 mM sodium phosphate, 140 mM NaCl, pH 7.4). Cell were plated at 60% confluence in normal (10% serum) medium. After attachment, the monolayers were wounded by scoring with a sterile plastic 1 ml micropipette tip. Each well was then washed and fed daily with normal medium. After 3 days, the cells were fixed with 0.2% glutaraldehyde in PBS for 5 minutes at room temperature and photographed using a low-magnification phase-contrast microscope. The extent of migration into the wound area was evaluated qualitatively.

25 *Immunofluorescence*

The cells were plated on fibronectin-coated slides (described above) for 20 minutes or 3 hours. They were then fixed 20 minutes with 4% (w/v) paraformaldehyde (PFA) in PBS and permeabilized with 0.1% (v/v) Triton X-100 in 4% PFA for another 30 minutes. The slides were blocked with 1% (w/v) bovine serum albumin (BSA) in PBS for 20 minutes. The cells were then incubated with the anti-vinculin antibody (1/400 dilution in PBS) for 1 hour at room temperature, washed three times with PBS and stained with a mixture of FITC-conjugated anti-mouse antibody (1/200) and 4 µl per well of TRITC-conjugated phalloidin (Molecular Probes Inc., Eugene, OR). After being washed 3 times with PBS, the coverslips were mounted in a 1:1 mixture of glycerol and 2.5% 1,4-Diazabicyclo [2.2.2]octane (DABCO) (Sigma) in PBS. The cells were visualized with a Nikon fluorescence microscope.

For cleavage furrow staining, the cells were plated at low confluency on uncoated glass slides and left overnight in 10% serum-containing medium. The cells

were then fixed, permeabilized and blocked as described above. Rhodamine-phalloidin was added at 4 μ l per well for 1 hour, then washed and mounted as described above.

Protein immunoprecipitation and immunoblotting

- Dishes of cells were washed with PBS supplemented with 1mM of sodium vanadate, harvested, lysed, and evaluated for protein content using the Bio-Rad Protein Assay (Hercules, Ca). For the immunoprecipitations of paxillin and cortactin, the cells were lysed in RIPA buffer (50 mM Tris-HCl pH7.2, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P40). For FAK, PSTPIP and PSTPIP2 immunoprecipitation, the cells were lysed by sonication in HNMETG (50 mM HEPES pH 7.5, 150 mM NaCl, 1.5mM MgCl₂, 1mM EGTA, 1% Triton X-100, 10% glycerol). For vinculin and p130cas, the lysis buffer used was TNE buffer (10 mM Tris HCl pH7.5, 1mM EDTA, 150 mM NaCl, 1% Nonidet P40). All the lysis buffers were supplemented with 1mM of sodium vanadate and COMPLETE protease inhibitor mixture (Boehringer Mannheim).
- For each immunoprecipitation, 500 μ g of total cell lysate was incubated with 1 μ g of antibody and 30 μ l of protein-G Agarose (Gibco BRL) in 1 ml of their respective lysis buffer (supplemented with vanadate and protease inhibitors) at 4°C for 90 minutes. The beads were then washed 3 times (the last wash for 15 minutes at 4°C) in their respective buffers, except FAK, PSTPIP and PSTPIP2 which were washed in HNTG buffer (10mM HEPES pH7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol), and resuspended in 30 μ l of SDS-PAGE loading dye.

The western blots were performed following common procedures; the proteins were transferred on a PVDF membrane, blocked and probed with the corresponding antibody in the same blocking buffer (1% BSA, 1% (v/v) goat serum, 0.1% Tween, in PBS). The following dilutions were used for the primary antibodies: 1:3000 for 4G10; 1:10,000 for anti-paxillin; 1:1000 for anti-vinculin, anti-p130cas, anti-cortactin and the HRP-conjugated pY20; 1:100 for anti-FAK. The secondary antibodies were used at a 1:10,000 dilution. The Renaissance™ chemiluminescence kit (NEN™ Life Science Products, Albany, New York) was used for detection.

Results

PTP-PEST-/- cells have a decreased motility on fibronectin

Two recent reports suggested an indirect correlation between p130cas phosphorylation level and cell migration rate. In both of these articles, a kinase was transfected in the cells; one of them used v-Src, for which p130cas is a direct substrate (14), while the other overexpressed FAK, that, once activated, binds both c-Src and its substrate p130cas (13).

Since p130cas is a substrate for PTP-PEST both in humans (6) and mice, and is constitutively hyperphosphorylated in cell lines where PTP-PEST is removed by gene targeting, we decided to investigate whether the absence of this PTP could

change the motility of these cells in the conditions where FAK and p130cas are activated.

The two cell lines used are hetero- and homozygotes for the PEST deletion. In order to minimize any dominant negative effects from the targeted allele, comparisons 5 were made between the homozygote and the heterozygote cell lines.

One of the simplest assay to qualitatively compare cell migration is the woundhealing migration assay. Fibroblast monolayers plated on overnight fibronectin-coated slides were wounded at 37°C and fixed after 36 hours. Figure 4 shows a typical aspect of the wound for each cell line obtained after five independent 10 experiments. The PEST (+/-) cells were able to migrate into the wound at a speed greater than the front of cells pushed in by proliferation (figure 4, panel A), whereas the (-/-) cells show a complete absence of chemokinetism (figure 4, panel B) and were not able to actively invade the wounded area.

PTP-PEST -/- cells have an increase in number and size of focal adhesions

15 The cytoskeleton of the cell plays an important role in cell motility. We then investigated whether this observed migration impairment of homozygous cells could be caused by an abnormal organization of the actin filaments or by a visible difference in focal adhesions. Cells from each cell line were plated on fibronectin. The actin 20 filaments were stained with a rhodamine-conjugated phalloidin, and the focal adhesions were highlighted by indirect immunofluorescence, using a mouse monoclonal antibody against vinculin and a fluorescin-conjugated anti-mouse secondary antibody.

After 25 minutes, both cell lines showed membrane ruffles and filopodia, suggesting that the defect in migration is not caused by an incapacity to polymerize 25 actin or to organize these structures. Large, immature focal adhesions were also found in each cell line, showing that the initial pathways forming these contacts are intact in (-/-) cells. (figure 5a-d)

However, when cells were left for 3 hours on fibronectin before fixing and staining, the homozygous cells remained well spread, with big and numerous focal 30 adhesion scattered throughout the ventral surface of the cell. Surrounding the cells are small, hair-like actin fibers that characterize cells in the early stages of apolar migration. The (+/-) cells, in contrast, are rounded, with well-defined edges and fewer focal contacts concentrated at the cell periphery. (figure 5e-h)

Constitutive hyperphosphorylation of p130cas and paxillin in the PEST -/-cell line

To understand the differences observed in the size and number of focal adhesions between the two cell lines and since they differ only by the presence of PTP-PEST, we then verified the tyrosine phosphorylation state of specific focal adhesion proteins (Figure 6).

The adapter protein p130cas was already shown to be hyperphosphorylated, since it is a substrate for PTP PEST and is included here as a positive control.

The other proteins immunoprecipitated and probed with an anti-phosphotyrosine antibody include cortactin and paxillin. Paxillin was shown to be 5 hyperphosphorylated in the FAK knock-out, a knock-out that was also associated with a decrease in cell mobility and increase in focal adhesions (33). Paxillin phosphorylation was also recently shown to be required for cell spreading and focal adhesion formation (46). In this case also, paxillin was found in a hyperphosphorylated state. Paxillin was recently shown to associate with PEST (30), but whether it is a 10 direct or indirect substrate of the PTP is still under investigation.

Vinculin is a structural protein that links talin and actin when focal adhesions form. It can be tyrosine phosphorylated, but this phosphorylation does not seem to be implicated in focal adhesion formation, which is rather due to a conformational change 15 in vinculin following phosphatidyl inositol biphosphate binding (12). There was no detectable basal level of tyrosine phosphorylation of vinculin in both cell lines, possibly due to the fact that the cells were not stimulated. (data not shown).

The last focal adhesion component to be investigated was FAK. The role of p130cas in migration was shown to reside in the pathways triggered by FAK (13), which is associated with the integrins and becomes active by trans-phosphorylation 20 when they cluster. This phosphorylation of Y397 on FAK, which is the binding site of the Src kinase, was shown to be crucial for its role in cell migration. Figure 6 shows that FAK is not hyperphosphorylated in the PEST knock-out cells.

Hyperphosphorylation of PSTPIP in the PEST -/- cells and effects on cleavage furrow formation

25 While we investigated the effects of PEST targeting in cell migration, another protein involved with the actin cytoskeleton, PSTPIP, was cloned and shown to be a substrate for a phosphatase of the PEST family, PTP HSCF (16). A putative coiled-coil region of PSTPIP interacts with the C-terminal, proline-rich region of PTP HSCF, a region that is present in all members of the PEST family, including PTPPEST.

30 Using a polyclonal antibody raised against PSTPIP, we verified its tyrosine phosphorylation level in the PEST (-/-) cells by immunoprecipitation, followed by an antiphosphotyrosine western blotting. Figure 7 shows that PSTPIP is hyperphosphorylated in the knock-out cell line, but whether PIP is a direct or indirect substrate is still under investigation. The same experiment was performed on 35 PSTPIP2, a protein with high homology with PSTPIP that does not contain the SH3 domain. The phosphorylation level of PSTPIP2 was similar in the +/- and the -/- cell lines (data not shown).

PSTPIP is homologous to *Schizosaccharomyces pombe* CDC15, an actin-associated protein that is involved in the formation of the cleavage furrow during cell

division (47). During our manipulations of the PEST (-/-) cells, we observed a high occurrence of cells that seemed blocked in cytokinesis, with the two daughter cells almost independent, but still attached to each other by an actin-rich junction. This phenomenon was postulated to be the result of a longer M phase due to an impairment 5 of the actin rearrangement during division. This phenomenon, although not quantified, was frequent in dividing -/- cells. Figure 8 shows the actin cleavage furrow ring as stained by rhodamine-phalloidin.

Discussion

Focal adhesion kinase (FAK) activation and focal adhesions formation are 10 closely related events. Until recently, the exact order in which they occur following integrin stimulation was greatly debated. Recent experiments (48, 49) showed that FAK activation is a result of the focal adhesion formation: due to the physical tension caused by the stress fiber formation in a cell, under the control of the rho GTPase, the associated integrins cluster and associate with other structural proteins like α -actinin 15 and tensin (12). Since the FAK proteins are associated with the integrin, they then become in close proximity to each other and can trans-autophosphorylate in a way similar to receptor tyrosine kinases following extracellular ligand binding. This phosphorylation activates FAK and provides docking sites for other signal transduction protein that propagate all the integrin-triggered pathways.

20 However, this relation between focal adhesion formation and FAK activation is not strictly linear. Amongst the signals sent by FAK, some were shown to feed back and play a role in the turnover rate of focal adhesions. The existence of such signal were concluded when the FAK knockout cell lines showed an increase in focal 25 adhesion size and number (33). Recently, FAK was shown to play a role in migration, an event that requires focal adhesion formation. This increase in migration occurs via the tyrosine phosphorylation of an adaptor protein, p130cas (13).

We examined another cell line with a targeted gene, this time the cytosolic 30 protein phosphatase PEST. Its main substrate both in human and mice was shown to be p130cas (6), and we recently showed that PEST can translocate to the membrane periphery following integrin activation, most probably in order to be able to reach its substrate which also translocates to focal adhesion when FAK is activated (9).

The effects of PTP-PEST removal on cell migration and focal adhesion size and 35 number were similar to the phenotype of the FAK knock out. Both showed a decrease of cell migration using fibronectin as a extracellular matrix, and the presence of large, immature focal adhesions scattered on the ventral face of the cell (Figures 4 and 5). Even though FAK is a kinase and PTP-PEST is a phosphatase with a common substrate, p130cas, PEST does not seem to antagonize the effects of FAK in cell migration, but rather to potentiate these effects. This suggests the presence of a

mechanism where both a formation and a breakdown pathway are triggered at the same time to increase the turnover rate of the focal adhesion structures. Without the pathway required for focal adhesion breakdown, the contacts between the extracellular matrix and the cell are too strong, and the cell adheres and does not move (50).

5 Interestingly, the FAK knock-out cells did not contain any hypophosphorylated focal adhesion components, but rather, like in this PEST knock-out, hyperphosphorylated proteins. The FAK knock-out showed hyperphosphorylation of cortactin and paxillin (p130cas was not tested). From these two, in the PEST knock-out, only paxillin is found in an hyperphosphorylated state, as well as p130cas which is its physiological substrate.

10 The phosphorylation of paxillin was shown to be associated with cell spreading on an extracellular matrix (46). It can associate to PEST (30), but whether it constitutes a physiological substrate for this phosphatase is still not clear.

15 It is not known exactly what role PEST plays in cell migration and focal adhesion formation. The absence of differences in the early stages of cell attachment to fibronectin (fig 6 a-d) suggests that PEST is not implicated in the formation of these structures. However, at equilibrium, differences appear (fig 3 e-h). The focal adhesions in the knockout cells are big and arrow head-shaped, which is a characteristic of immature focal adhesions. They are numerous and scattered throughout the ventral membrane of the cell, which can explain their absence of motility when tested in the wound healing assay. In contrast, the heterozygote cells became rounded, and their focal adhesions are punctual and found only at the tips of their membranes, which is consistent with a cell that is migrating. This led us to believe that PEST is implied in focal adhesion breakdown but whether PEST actively breaks down the focal adhesion structures or normally shuts down a positive feedback for focal adhesion formation is not clearly demonstrated here.

20 Studies using the tyrosine phosphatase inhibitor phenylarsine oxide showed that treatment of cells with this compound was sufficient to induce the formation of stress fibers even after starving them for 16 hours. They also showed that focal adhesion disassembly results in stimulation of phosphatase activity, which could be assayed using FAK and paxillin as substrates. Phenylarsine oxide reacts with two thiol groups of closely spaced cysteine residues in the active site of the phosphatase. The PTP-PEST catalytic domain contains the sequence ²³¹CSAGC²³⁵ (3; SEQ ID NO: 3), the cysteine at position 231 being crucial for its catalytic activity. That and the fact that 25 paxillin is hyperphosphorylated in the PEST knock-out cell line suggest that PTP-PEST is a candidate phosphatase involved in the focal adhesion breakdown in the conditions studied in these experiments, and that this role can be put in parallel with its role deduced above in cell migration.

30 This model for the role of a PTP in focal adhesion breakdown would suggest

that overexpression of the phosphatase would also inhibit cell migration, but this time by impairing the formation of the focal adhesions at the leading edge of the cell. Indeed, experiments involving another PTP that can dephosphorylate p130cas, PTP1B (37), showed that overexpression of this phosphatase in rat fibroblasts decreased cell 5 migration while increasing the time required for the cell to spread on fibronectin (51). This was linked to a disordered formation of focal adhesions. Interestingly, these cells eventually formed numerous, large focal complexes scattered over their ventral surface, like the ones found in the PTP-PEST-/- cells. These experiments and the ones presented in this paper suggest that an intermediate level of PTP activity towards 10 p130cas is required for the formation of normal focal adhesions and for cell migration, which is consistent with a role in focal adhesions turnover.

PTP-PEST may also play another role in the regulation of the cell cytoskeleton, this time via the cleavage furrow-associated protein PSTPIP. PSTPIP was originally identified as a binding partner and a substrate for the phosphatase PTP-HSCF (16), 15 a PEST tyrosine phosphatase. PTP-HSCF dephosphorylates tyrosine residues in PSTPIP that are modified either by co-expression of the v-Src tyrosine kinase or in the presence of the unspecific PTP inhibitor pervanadate. One of these sites, within the SH3 domain of PSTPIP, was shown to regulate the binding with the proline-rich region found on the Wiskott-Aldrich syndrome protein, WASP (52) and to control aspects of 20 the actin cytoskeleton.

It has not been shown whether PTP-PEST binds directly PSTPIP, but peptides derived from the C-terminus of PEST can compete the binding of PTP-HSCF to PSTPIP (16). Also, no experiments show that PTP-PEST can dephosphorylate PSTPIP, but the fact that PSTPIP is hyperphosphorylated in the PEST-/- cells (Figure 25 7) suggest that PTP-PEST plays a role in modulating the phosphorylation level of PSTPIP. PTP-HSCF and another member of the PEST family, PTP-PEP, are not expressed in fibroblasts, and it is possible that PTP-PEST plays on PSTPIP a role in fibroblasts equivalent to PTP-HSCF in hematopoietic cells. The exact site that is hyperphosphorylated on PSTPIP is not known, and the effects of this 30 hyperphosphorylation are still under investigation. One of them appears to be an increase in the relative amount of cells found in M-phase in a field of unsynchronized cells (Figure 8), possibly due to the role of PSTPIP in cleavage furrow formation. However, since the cultures of -/- cells grow at a rate comparable to the +/- cells, this effect appears to be secondary. It is also possible that this increase of PSTPIP tyrosine 35 phosphorylation is the result, and not the cause, of the increase of the number of cells in M-phase. But since the hyperphosphorylation of PSTPIP is much more evident than the increase of cells in metaphase, the opposite is more likely.

In this article, we reported two roles that a specific PTP, PTP-PEST, could play in regulating the cytoskeleton of fibroblasts. The first one, possibly via its capacity to

dephosphorylate p130cas, is to break down focal adhesions, an event which is also required for cell migration on an extracellular matrix like fibronectin. PTP-PEST was shown to localize at the membrane periphery when COS cells were plated on fibronectin and this confers to PTP-PEST a physiological role in cell migration, one that
5 is not a secondary effect caused by overexpression. The fact that PTP-PEST is mostly found in a cytoplasmic pool (3) and can be recruited at will provides the cell a way to increase its focal adhesion turnover rate proportional to the stimulus, even when FAK,
10 p130cas or v-Src are overexpressed (13, 14, 53). PTP-PEST also plays a role in modulating the phosphorylation level of PSTPIP, a protein that associates with the cytoskeleton (16). The role of this PTP-PEST activity is not known, whether it involves the binding of WASP to PSTPIP, the role of this latter in cleavage furrow formation or any other yet unknown function that PSTPIP possesses in fibroblasts.

EXAMPLE 3:

THROUGH THE IDENTIFICATION OF PUTATIVE SUBSTRATES OF PTPs:

15 The fibroblast cells that we generated from knock out mice having a gene targeted PTP-PEST (-/-) was used to identify potential substrates with the premise that specific and physiological substrates of this enzyme would exist in a hyperphosphorylated state. Analysis of the phosphotyrosine profile of the -/- cells revealed the presence of hyperphosphorylated p180, p130 and p97. Since the protein
20 p130cas was isolated from pervanadate treated cells using substrate trapping mutants of PTP-PEST (6), we have analysed its tyrosine phosphorylation status. Direct immunoprecipitates of p130Cas demonstrate that this protein is hyperphosphorylated in the -/- cells. In addition, a strong interaction between PTP-PEST and SH3 domain of the Cas-like molecule Hef1 and Sin was observed *in vitro*. By combining genetic and
25 biochemical strategies, we identify putative substrate of PTP-PEST. Indeed, we demonstrate for the first time that the Cas-like molecules Hef1 and Sin associate with a proline rich motif on PTP-PEST via their SH3 domains and are thus potential substrate. Based on these results, the combination of gene targeting of a PTP and substrate trapping approaches can be reasonably applied to the identification of PTP-
30 PEST and of other PTPases.

During the search for PTP-PEST targets, the adaptor protein p130cas was identified as a substrate for PTP-PEST by substrate trapping experiments (6). Two "substrate trap" mutants, PTP-PEST D199A and C231S, have been used to isolate the unique substrate p130cas from pervanadate treated HeLa cell extracts. In a murine context, ligand dependent activation of the EGF receptor provides a way to induce the association of PTP-PEST C231S "substrate trap" mutant with a pp130 kDa protein not yet identified, but most likely p130cas (3). The recognition of the tyrosine phosphorylated p130cas in pervanadate treated cell extracts by PTP-PEST D199A was recently shown to be severely impaired by the mutational inactivation of a proline
35

rich region (residue P³³⁷) and the authors proposed a mechanism for PTP-PEST substrate recognition via the SH3 domain of p130cas (4).

p130cas is an adaptor protein composed of an SH3 domain, 2 proline rich regions, a substrate domain for tyrosine kinases containing 15 YxxP sites (54, 56) and 5 a binding site for the SH2 domain of the Src kinases (YDYV motif). The SH3 domain of p130cas has been shown to interact with proline rich sequences found in FAK, FAK related nonkinase (FRNK) and PTP1B (37, 54, 55). In a recent study, the SH3 domain of p130cas was found essential for targeting p130cas to focal adhesions in non-transformed cells (9). The substrate domain of p130cas can interact in a 10 phosphotyrosine dependent manner with the SH2 domains of v-crk (57), Crk (58), Crk-II (59), CRKL (60), Nck (61) and several other yet unidentified SH2 domain containing proteins (57). p130cas has been shown to become tyrosine phosphorylated following treatment of cells with EGF (58), NGF (59), PDGF (62) and also by integrin 15 activation (63). The YDYV motif and the proline rich region at the C-terminus of p130cas are binding sites for the SH2 and SH3 domains of Src family of tyrosine kinases resulting in phosphorylation of other tyrosine residues on p130cas by these kinases (64).

Two other proteins share a high degree of homology with p130cas Hefl/Cas-L (65,66) and Efs/Sin (67,68). Because they have a similar modular structure to the one 20 found on p130cas, Hefl and Sin belong to the p130cas family of adaptor proteins. The SH3 domains of Hefl and Sin are respectively 74% and 64% identical to the SH3 domain of p130cas. The substrate domain of Hefl and Sin contains 13 and 8 YxxP motifs respectively. Many of these YxxP motifs are different than the motifs found in p130cas suggesting that Hefl and Sin could interact with different SH3 domain 25 containing proteins. Hefl and Sin expression is restricted to certain populations of cells, whereas p130cas is ubiquitously expressed. Hefl is expressed mainly in hematopoietic cells (66). Much less is known about Sin expression, although its mRNA is found at high levels in the mouse embryo (67).

The identification of physiological substrates of protein tyrosine phosphatases 30 is a key element in understanding the biological functions of this family of enzymes. The principal aim of this study was to describes a novel experimental approach in the identification of PTPs substrate(s) by combining the technology of gene targeting of a PTP and substrate trapping experiments using PTP-PEST as a paradigm. Using this approach, p130cas was isolated in high amount by a PTP-PEST trapping mutant thus 35 supporting the strategy. A second objective of the present study was to demonstrate for the first time that the other members of the p130cas family of proteins, Hefl and Sin, interact in a similar manner with a proline rich region found on PTP-PEST with their SH3 domains.

Materials and Methods

Generation of PTP-PEST gene targeted cell lines PTP-PEST +/- and -/- embryonic fibroblast cell lines were established from primary embryonic fibroblast cultures isolated from PTP-PEST heterozygous (+/-) and homozygous (-/-) mouse embryos respectively, generated by targeted disruption of the PTP-PEST locus by homologous recombination in ES cells¹. Briefly, 8.5 days after mating PTP-PEST (+/-) females with PTP-PEST (+/-) males, embryos were isolated trypsinized for 30 minutes at 37°C. Cells in suspension were isolated and cultured. At confluence, cells were split and immortalized by stably transfecting, using Lipofectamine (GibcoBRL), a dominant negative mutant p53 (C153S) gene (25) along with EIA/EIB viral genes (obtained from Dr. Branton, McGill University) and a vector conferring puromycin resistance. Cells surviving puromycin treatment were pooled and genotyped by Southern and Northern blot using standard techniques. For Southern blotting, the genomic DNA was digested with BamHI and separated on a 0.8% agarose gel. After transfer to Hybond N+, the blot was probed with a radiolabeled KpnI/SacI fragment of the PTP-PEST cDNA. For Northern blotting, the RNA was separated on a 1 % formaldehyde agarose gel and following transfer to Hybon N+, the blot was probed with a radiolabeled probe generated by PCR from the PTPase domain of PTP-PEST. The blot was stripped and probed with GAPDH to verify equal loading of RNA.

Constructs The p130cas mouse cDNA in pBluescript was a generous gift from Dr. Steven Hanks (Vanderbilt School of Medicine, Tennessee). The p130cas cDNA was cloned in pJFTAG, pBluescript II KS (Stratagene) and pCDNA3 (Invitrogen) in the EcoRI and Sa1I sites using standard recombinant DNA technology. pJFTAG is pCDNA3 in which six copies of the c-myc epitope were cloned in the HindIII and EcoRI sites. The SH3 domain of p130cas was cloned in the EcoRI and Apal sites of pJFTAG. The GST-SH3 domain of p130cas and Sin were constructed by amplifying the region encoding for the SH3 domains by PCR using oligonucleotides containing engineered BamHI and EcoRI sites followed by cloning of these products in the BamHI and EcoRI sites of pGEX 2TK (Pharmacia). The GST SH3 domain of Hef1 was cloned in pGEXILambdaT by Dr. Yuzhu Zhang and was a kind gift from Dr. Erica Golemis (Fox Chase Cancer Center, Philadelphia).

Substrate trapping experiments 100 ng of GST PTP-PEST aa 1-453 or GST PTP-PEST C231S aa 1-453 pre-bound to glutathione sepharose were incubated with 1 mg of lysate from the indicated cell extracts for 90 min as described previously (4). The beads were washed extensively in HNTG buffer and proteins were eluted in SDS-sample buffer. After SDS-PAGE and transfer to PVDF membrane, immunoblotting was performed with the antiphosphotyrosine monoclonal antibody 4G10 or a rabbit polyclonal anti-p130cas B+F (a generous gift from Dr. Amy H. Bouton, University of Virginia).

Cell culture, transfections and co-immunoprecipitation 293-T, COS-1,

PTP-PEST +/- and PTP-PEST -/- were maintained in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin. 293T cells were transiently co-transfected with 20 pg total of PTP-PEST and p130cas plasmids using the calcium phosphate precipitation method 5 as described in (5). Exponentially growing COS- 1 cells from a 15 cm tissue culture dish were also transfected with 40 µg of pACTAG PTP-PEST by electroporation as described in (3). For co-immunoprecipitations, 293T cells transiently expressing HA-PTP-PEST and Myc-p130cas proteins were harvested and washed with PBS. Cells were lysed in HNMTG as described in (5). Protein complexes were 10 immunoprecipitated using anti-PTP-PEST 1075 antibody (3) and protein G-agarose (Gibco-BRL) for 90 min at 4°C. Immune complexes were washed three times in HNTG and proteins were eluted from beads in SDS sample buffer by boiling for five minutes. Proteins were separated on a 11 % SDS-PAGE, blotted on PVDF membrane 15 (Immobilon-P, Millipore) and analyzed for the presence of the Myc tagged p130cas proteins using the anti-Myc epitope monoclonal antibody 9E10. COS-1 cell were treated with pervanadate in order to increase protein tyrosine phosphorylation levels. Briefly, 360 µL of freshly prepared pervanadate (10 mM sodium orthovanadate in 50 mM hydrogen peroxide) was added to 15 mL of D-MEM and cells were incubated for 15 min before harvesting.

20 *Binding studies* GST fusion proteins encoding the different portions of PTP-PEST have been described elsewhere (3). The GST fusion proteins were purified with glutathione Sepharose (Pharmacia) according to manufacturer's protocol. For liquid binding studies, 100 ng of GST alone or GST-SH3 domains fusion proteins prebound to glutathione Sepharose were incubated with 1 mg of COS-1 cell lysate 25 expressing HA-PTP-PEST for 90 minutes at 4°C. After washing three times in HNTG, the proteins were eluted in sample buffer, separated by SDS-PAGE and the presence of HA-PTP-PEST was analyzed by immunoblotting with anti-12CA5 antibody. For farwestern assays, purified PTP-PEST fusion proteins were separated on SDS-PAGE 30 and transferred to PVDF membrane. Proteins were allowed to renature on the blot overnight at 4°C in binding buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.2% BSA). Blots were probed with [³²p] labeled GST-SH3 domains fusion proteins (labeled according to manufacturer's instruction, Pharmacia) for 8 to 16 hours in binding buffer at 4°C. The blots were washed extensively in binding buffer at room temperature and exposed to X-ray film (Kodak) for 20 minutes.

35 Results

Generation of gene targeted PTP-PEST cell lines (PTP-PEST -/-) In order to study the phosphotyrosine profile content of cells lacking PTP-PEST with the aim of identifying novel substrate, we generated mouse embryonic fibroblasts from 8.5 day post-coitum embryos isolated from the mating of PTP-PEST+/- animals. These primary

cultures were immortalized with a intermediate transforming C135S mutant of p53 (69) and EIA/EIB proteins as described in the materials and methods section. The genotypes of the established cell lines were determined by Southern blotting. Figure 9a shows that one of the cell lines have both the 12 kb wt and 7 kb targeted allele (PTP-PEST +/-) while the other cell line only has the targeted allele (PTP-PEST-/-). As a positive control, wt DNA isolated from embryonic fibroblasts was included and only the wt allele was observed. In order to verify the absence of PTP-PEST mRNA in the PTP-PEST -/- cell line, a Northern blot analysis was performed. In figure 9b, the 3.8 kb mRNA of PTP-PEST is present in the PTP-PEST +/- but absent from the -/- cells (upper panel). As a positive control, RNA isolated from embryonic fibroblast was included and a 3.8 kb signal was observed. As a loading control~ the blot was stripped and probed with GAPDH (figure 9b, lower panel). The phenotypic characterization of the PTP-PEST deficient cell lines is presently in preparation.

Analysis of the phosphotyrosine profile of the PTP-PEST -/- cells In order to identify putative substrate(s) of PTP-PEST, we have analysed the phosphotyrosine profile of the PTP-PEST -/- cells. Comparison of antiphosphotyrosine immunoblotting of the total cell lysate (TCL) of the +/- and -/- revealed the presence of hyperphosphorylated proteins of approximately 180, 130, and 97 kDa in the -/- cells (Figure 10 a, first two lanes). When the TCL of the +/- and -/- cells were subjected to immunoprecipitation using an antiphosphotyrosine antibody and analyzed by antiphosphotyrosine western, the same hyperphosphorylated proteins were detected (Figure 10 a, last two lanes).

In order to identify these hyperphosphorylated proteins, an *in vivo* immunoprecipitation of candidate proteins were performed. p130cas was immunoprecipitated both from the PTP-PEST +/- and -/- cell lines and the phosphorylation status of p130cas was analyzed by antiphosphotyrosine immunoblotting using the 4G10 antibody. In the cells lacking PTP-PEST (-/-), p130cas was found to be hyperphosphorylated when compared to p130cas found in the PTP-PEST +/- cell (figure 10b, upper panel). Equal amounts of p130cas protein both in the immunoprecipitates and in the total cell lysates (TCL) was verified by stripping the blot and reprobing with a rabbit polyclonal anti-p130cas as shown in figure 2b (lower panel). Since we have previously demonstrated that PTP-PEST forms a complex with the EGF receptor via Grb2 (3), the amount of EGF receptor in antiphosphotyrosine IP from the +/- and -/- was compared. Equal amounts of EGF receptor were found in both IP (data not shown). As the putative 97 kDa protein(s), the amount of the p130cas-like proteins Hefl and Sin was also analyzed in antiphosphotyrosine IP from the +/- and -/- cells. Hefl and Sin were not found in these IP (data not shown). Furthermore, we have observed that Hefl and Sin are not expressed in the PTP-PEST +/- and -/- fibroblast cell lines used in the present study.

Combination of gene targeting of PTP-PEST and substrate trapping experiments to identify physiological substrate(s). The inactivation of the PTP-PEST gene (PTP-PEST $-/-$ cell line) was hypothesized to render substrate(s) that are highly specific to PTP-PEST in an hyperphosphorylated state and that these substrate(s) could subsequently be isolated using a "substrate trap" mutant of PTP-PEST (C231S mutant). An important aspect of this novel approach is that, before performing the trapping, the cells don't require physiological (growth factor) or artificial (pervanadate) treatments. A substrate trapping experiment was thus performed in PTP-PEST $-/-$ and $+/-$ cell lysates using a GST PTP-PEST C231S mutant. As shown in figure 11 (panel A), the GST PTP-PEST WT was unable to isolate phosphorylated substrates both in the PTP-PEST $+/-$ and $-/-$ cell lysates whereas the C231S mutant isolated a small amount from the PTP-PEST $+/-$ cell lysates and a abundant amount from the PTP-PEST $-/-$ cell lysate of phosphorylated pp130 proteins. The same substrate trapping experiment was performed, as a positive control, using lysate of pervanadate treated COS-1 and a pp130 protein was also isolated with the PTP-PEST C231 mutant. Figure 11 (panel B) shows the same blot stripped and analyzed for the presence of p130cas protein using anti-p130cas B+F. p130cas proteins were found mostly in the lanes of PTP-PEST C231S mutant in PTP-PEST $-/-$ and pervanadate treated cell lysate. Interestingly, the same amount of p130cas proteins were affinity purified from the PTP-PEST $-/-$ cell lysate and the pervanadate treated COS- 1 cell lysate by the C231S mutant of PTP-PEST (figure 11, panel C), but the tyrosine phosphorylation level of p130cas trapped from the PTP-PEST deficient cell line is approximately twenty times greater, as estimated using densitometry, when compared to p130cas trapped from the pervanadate treated extract.

PTP-PEST associates with the SH3 domains of the p130cas-like proteins Hefl and Sin. The SH3 domain of p130cas was demonstrated to interact with PTP-PEST in the substrate recognition mechanism (7). The presence of SH3 domains on Sin and Hefl, p130cas-like proteins, have prompted us to investigate if these SH3 domains could interact with PTP-PEST. The SH3 domains of p130cas and Sin were cloned in pGEX-2TK and the SH3 domain of Hefl was cloned in pGEXILambdaT vector and expressed as GST fusion proteins in order to perform a liquid binding assay with PTP-PEST. The integrity of the fusion proteins used in the binding assay is shown on a coomassie blue stained gel in the right panel of figure 12. As seen in figure 12 (left panel), HA-PTP-PEST was specifically bound to the purified SH3 domains of Hefl and Sin even after extensive washing of the complex. GST alone was used as a negative control and did not bind the HA-PTP-PEST while used as a positive control, the SH3 domain of p130cas was bound to HA-PTP-PEST.

Mapping of the first proline rich region of PTP-PEST(amino acids 316-346) as the binding sites for the SH3 domains of Sin and Hefl. Because five proline rich motifs

are found on PTP-PEST (3) and SH3 domains are known to bind to proline rich motifs, we investigated which one of these motifs is mediating the interaction with the SH3 domains of Hefl and Sin. A series of GST fusion proteins encoding for the five different proline rich regions of PTP-PEST (Pro 1-5) was generated in order to map the region essential on PTP-PEST for the interaction with the SH3 domains of the Cas-like proteins Sin and Hefl in a farwestern binding assay. A schematic representation and a coomassie blue stained gel of the fusion proteins showing integrity of the products used in the farwestern assay are shown in figure 13a and 13b respectively. For the farwestern assay, the PTP-PEST fusion proteins were separated by 11% SDS-PAGE and then transferred to PVDF membranes and after blocking, the blot was probed with [³²p] radiolabeled SH3 domains of either Sin, Hefl or p130cas. The SH3 domains of Sin and Hefl were found to associate with high affinity to the Pro1 region of PTP-PEST (aa 316-346 SEQ ID NO: 4 or aa 317-347 (SEQ ID NO: 5) of mouse or human PEST, respectively) and also to the complete C-terminus (aa 276-775) and to the N-terminus (aa 1-453) fusion proteins of PTP-PEST as seen in figure Sd and e. These three proteins have in common the Pro1 region of PTP-PEST. No detectable levels of binding of these SH3 domains to the Pro 2-5 of PTP-PEST and GST alone (negative control) were noted even after a longer exposure. The SH3 domains of p130cas was used as a positive control to probe an identical blot and the same result was obtained (figure 13a). These results demonstrate that the SH3 domains of the family of adaptor proteins p130cas, Sin and Hefl interact with high affinity and selectively with the Pro1 region of PTP-PEST.

PTP-PEST and p130cas associate in vivo. The association between PTP-PEST and p130cas has only been investigated *in vitro* using either GST fusion proteins (present work) or baculovirus produced proteins (6, 11). In order to evaluate the binding between p130cas and PTP-PEST *in vivo*, we performed a co-immunoprecipitation experiment. 293T cells were co-transfected by the calcium phosphate method with HA-PTP-PEST WT or C231S (10 µg) and either Myc-p130cas or Myc p130cas SH3 domain (10 µg)- A schematic representation of the p130cas proteins is shown in figure 14a. The cell lysates were immunoprecipitated with anti-PTP-PEST 1075 and analyzed for the presence of Myc p130cas proteins (figure 14b). The Myc-p130cas and the Myc-p130cas SH3 domain were found in the HA-PTP-PEST IPs (figure 14b). As a negative control, the Myc p130cas and Myc p130cas SH3 domain were cotransfected with HA-T-cell PTP (TC-PTP). Figure 14b demonstrate that neither Myc p130cas nor Myc p130cas SH3 domain were found in the TC-PTP immunoprecipitation clearly showing that this interaction is specific for PTP-PEST. These results demonstrate that the association of PTP-PEST occurs *vivo* with the full length p130cas or specifically with the SH3 domain of p130cas.

Discussion

The identification of protein tyrosine phosphatase substrates is one of the key steps in understanding the biological functions of this family of enzymes. Recent mutagenesis experiments of invariant amino acids within the conserved catalytic domains of PTPs, in conjunction with the analysis of the crystal structure of PTP1B, 5 have yielded two interesting mutants for substrate trapping: First, a cysteine to serine mutant in the VHCSAG (SEQ ID NO: 6) signature motif (C215S in PTP1b) and second, a mutation of a catalytically active aspartic acid to alanine (D199A in PTP1b) (2). The present data propose a novel approach by combining substrate trapping experiments with the gene targeting of a PTP, PTP-PEST, to identify putative physiological 10 substrate(s). Fibroblasts cell lines lacking PTP-PEST were generated from the PTP-PEST knock-out¹ and used as protein extracts in a substrate trapping experiments. Using such an approach, p130cas was the main protein isolated by a PTP-PEST substrate trapping mutant. The advantage of this novel approach is that only physiological substrate(s), since cells are not treated artificially to increased their 15 phosphotyrosine contents, that are highly specific for the PTP of interest would possibly be hyperphosphorylated in the gene targeted tissues or cell lines. As a result, mainly such substrates should be isolated in large amounts by the substrate trapping mutants of the chosen PTP.

It stands to reason that the removal of a PTPase will result in the 20 hyperphosphorylation of its physiological substrates at the basal level or following specific stimulation. For example, it was shown that when the vaccinia virus dual specificity phosphatase VH1 is deleted from virion capsule two encapsidated substrate proteins, p18 and p11 become hyperphosphorylated and as a result, the life cycle of the virus is impaired (70). With the premise that physiological substrate of PTP-PEST 25 would become hyperphosphorylated, we generated a gene targeted PTP-PEST fibroblast cell line, PTP-PEST-/- . In this case, the antiphosphotyrosine profile of the total cell lysate of PTP-PEST-/- cell line was compared to the total cell lysate of PTP-PEST+/- and hyperphosphorylated proteins of 180, 130 and 97 kDa were identified in the -/- cells. One of these proteins was identified as p130cas. Direct 30 analyses by antiphosphotyrosine blotting of p130cas immunoprecipitates from PTP-PEST +/- and -/- clearly indicate that p130cas is hyperphosphorylated in the absence of PTP-PEST. This suggests that even if many PTPs are expressed in these murine fibroblasts, and that at least one of them, PTP1B, has been shown to interact 35 with the SH3 domain of p130cas (37), the major regulation of the phosphorylation of p130cas at the basal level is mediated by PTP-PEST. The identity of the hyperphosphorylated p97 and p180 is still under investigation. We have investigated if the p180 protein could be the EGF receptor since we have previously shown that PTP-PEST can complex with the EGF receptor via Grb2 (4). The EGF receptor was found in equal amount in antiphosphotyrosine immunoprecipitation in the PTP-PEST

-/- cells and +/- cells. Hefl and Sin were possible candidates for the hyperphosphorylated p97. Both of these proteins were not expressed in these fibroblast cell lines. This observation is not surprising since Hefl and Sin have a restricted pattern of expression.

5 The data presented in this paper also describes that the family of adaptor proteins p130cas, Sin and Hefl interact with PTP-PEST via their SH3 domains. The first proline rich region (Pro1) of PTP-PEST, ³³³PPKPPR³³⁸ (SEQ ID NO: 7), was found to mediate the interaction with the SH3 domains of the latter family of proteins without contribution of the four other proline rich motifs (Pro 2-4) found on PTP-PEST. Our
10 data support recent findings by Garton et al. (4) suggesting that the SH3 domain mediated association of p130cas with PTP-PEST potentially play a crucial role in the substrate specificity. These results also suggest both Hefl and Sin are candidate substrates for PTP-PEST since they also associate with PTP-PEST via their SH3 domains. It would of been interesting to analyze the phosphorylation status of Hefl and
15 Sin in the PTP-PEST -/- cells, but unfortunately, they were not expressed.

The proline rich region (Pro1) of PTP-PEST, ³³³PPKPPR³³⁸, belongs to the class 2 of consensus sequence (PxxPxR) (71). Other proline rich domains that have been shown to interact with the SH3 domain of p130cas also belong to the class 2 of consensus sequence: PRPPKR for PTP 1B (37), PPKPSR for FAK and the RAFTK (55, 72). Three other enzyme share homology to PTP-PEST: PTP-PEP, PTP-HSCF and PTP-BDP. Table 1 summarizes the putative proline rich region found on this family of PTP. PTP-PEP has two identical proline rich domain having the sequence PPLPER and thus belongs to the class 2 of consensus sequence (73). It is unlikely that the two proline rich sequences of PTP-PEP could interact with the SH3 domains of p130cas, 25 Sin or Hefl since an identical proline rich sequence found on PTP-PEST (Pro 4, ⁶⁷⁵PPLPER⁶⁸⁰; SEQ ID NO: 8) does not interact with these SH3 domains (this paper, figure 5). Analysis of the PxxP motifs found on PTPHSCF and PTP-BDP (Table 1) reveals that none of them belong to the class 2 of consensus sequence (PxxPxR) and in this respect, these two PTPs probably do not interact with the SH3 domain of
30 p130cas, Sin and Hefl. In summary, among the member of PEST like PTPs, PTP-PEST appears to be the only one that can interact with the SH3 domain containing proteins p130cas, Sin and Hefl via a proline rich region. The association of PTP-PEST to the SH3 domain of p130cas have been demonstrated to play a crucial role in the substrate recognition mechanism (11). Since PTP-PEST is expressed ubiquitously and p130cas, Hefl and Sin have differential expressions, it is reasonable
35 to envisage that PTP-PEST is a potential modulator of the tyrosine phosphorylation level of this family of adaptor proteins in different cell types.

We have clearly demonstrated that PTP-PEST, WT or C231S, is constitutively associated with p130cas in an *in vivo* coimmunoprecipitation experiment and that the

SH3 alone of p130cas can mediate this interaction. In this respect, the proline rich/S_H3 domain association is constitutive as usually observed in this kind of interaction and does not terminate when p130Cas becomes dephosphorylated by the catalytic activity of PTP-PEST as previously proposed (6). The deletion of the Pro1 domain of PTP-PEST completely abolishes the binding with the SH3 domain of p130cas (4), Hef and Sin *in vitro* thus clearly demonstrating an essential binding site between these proteins. Interestingly, the association between PTP-PEST and p130cas was not abrogated by the deletion of the Pro 1 domain of PTP-PEST and/or the SH3 domain of p130cas in a co-immunoprecipitation experiment performed in transfected 293-T cells. These results suggest that PTP-PEST and p130cas might also associates via a SH3 independent mechanism and this is presently under investigation.

In summary, the combination of gene knock-out of a PTP and substrate trapping experiments is potentially a powerful approach to identify relevant physiological substrate(s) of a given PTP. Using such an approach, p130cas is confirmed as a physiological substrate of the murine PTP-PEST. We believe that this approach reduces the risk of artifacts since no artificial treatments of the cells is required and that it could be used to identify substrate(s) of other PTPs. The SH3 domains of the p130cas-like molecules, Sin and Hefl, were found to interact with high affinity with a proline rich region of PTP-PEST (Pro1, aa 333-338 (SEQ ID NO: 7)). These data also suggest that Hefl and Sin could be candidate substrates for PTP-PEST. This hypothesis is also supported by the fact that an effective dephosphorylation of p130cas by PTP-PEST requires an intact Pro 1 region. The *in vivo* association of PTP-PEST and p130cas has been demonstrated to be stable and constitutive by coimmunoprecipitation, which was not clear from a previous report (6).

25 **EXAMPLE 4**

PTP-PEST BINDS TO PAXILLIN THROUGH ITS PRO 2 REGION:

Since paxillin is hyperphosphorylated in PEST *-/-* cells, we further investigated which respective domains of each molecule interact. We also confirmed that paxillin, although capable of binding to PEST, is not a substrate for this phosphatase. Paxillin is a member of a family of adaptor proteins that also includes Hic 5 (17) and leupaxin (18). Located in focal adhesions, paxillin associates with important cytoskeletal proteins such as talin and vinculin as well as protein tyrosine kinases found in adhesion plaques such as p125FAK, Pyk2 and c-Src (19). In particular, the association of p125FAK with paxillin has been shown to be essential for focal adhesion targeting of p125FAK (20). In addition, following integrin engagement, paxillin has been demonstrated to be phosphorylated by p125FAK and c-Src (21,22). This creates docking sites for the SH2 domain of the Crk proteins (23) and links the integrin activation to signal transduction pathways via the proteins C3G or SOS that are bound to Crk. In addition to its + tyrosine phosphorylation, paxillin is also heavily

phosphorylated on serine and threonine residues following plating of cells on fibronectin (24). Serine and threonine phosphorylation of paxillin have been implicated in its targeting to focal adhesions and cellular attachment to fibronectin (25).

- Structurally, paxillin and the paxillin-like proteins are composed of N-terminal LD motifs and four C-terminal LIM domains. The LD motifs of paxillin (reviewed in (26)) have been shown to be implicated in the paxillin binding to p125FAK and vinculin (19). LD motifs have also been observed in a variety of proteins where they also act as mediating protein-protein interaction (26). LIM domains are approximately 50 amino acids in length and known to mediate protein-protein associations (for review (27,28)).
- 5 The LIM domains have a conserved consensus sequence: [CXXC(X₁₆₋₂₃)HXXC]XX[CXXC(X₁₆₋₂₁)CXXC/D/H] (28). Proteins harboring LIM domains often harbor other domains such as homeodomain, kinase, SH3 and LD domains. One of the most characterized LIM domain mediated interaction involves the association of the LIM 3 of Enigma to the tyrosine based motif (tyrosine tight turn) of the insulin receptor (29). Similarly, the LIM 2 of Enigma interacts with the Ret receptor tyrosine kinase (29). These interactions indicate that each LIM domain may indeed recognize and interact with specific protein domains. The LIM domains of paxillin, especially LIM 15 3, are essential for proper focal adhesion targeting. Although the focal adhesion targeting ligand of LIM 3 has not been identified (19), it has recently been shown that 20 LIM2 and LIM3 bind protein(s) with serine kinase activity (25).

In a recent report, paxillin was shown to associate directly with the C-terminus tail of PTP-PEST by a yet uncharacterized mechanism (30). In the present study, we reveal that a non-classical proline rich motif of PTP-PEST (Pro 2) is essential for both *in vitro* and *in vivo* binding of PTP-PEST to paxillin. More precisely, mutation of proline 25 362 to alanine completely abolishes this association. The presence of intact LIM3 and LIM4 domains of paxillin were required for its association with PTP-PEST. Finally, using mutants of PTP-PEST having a C231S mutation, we demonstrate that paxillin is not a substrate for PTP-PEST in a substrate trapping assay. Together, these results demonstrate a novel association between LIM domains and a proline rich motif. We 30 propose that a physiological function for this association could be that once PTP-PEST is recruited to adhesion plaques, it could regulate the breakdown and turnover of focal adhesions by dephosphorylating and/or binding proteins such as paxillin, p130Cas, Shc and Grb2.

Materials and methods

- 35 **Cell lines, transient transfections and pervanadate treatment** NIH 3T3, NIH 3T3 overexpressing a Src Y527F constitutively active mutant and HEK 293T cell lines were routinely maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (Gibco-BRL). HEK 293T cells were transfected with 5 µg of PTP-PEST plasmid using the calcium phosphate technique

as previously described (10). NIH 3T3 cells were pervanadate treated for 30 minutes as previously described (10).

- Antibodies** The monoclonal antibodies against paxillin (P13520) and p130Cas (P27820) were from Transduction Laboratories. The polyclonal antibody specific for avian paxillin was previously described (19). The anti-GST and anti-HA tag antibody 12CA5 were obtained from Santa Cruz Biotechnology. The antiphosphotyrosine antibodies 4G10, PY20 and PY99 were from Upstate Biotechnologies, Transduction Laboratories and Santa Cruz Biotechnology respectively. The polyclonal antibody 1075 against PTP-PEST was previously described (3).
- Plasmids construction** PTP-PEST cDNA in the expression vector pACTAG (HA epitope tagged) was previously described (3). The Pro 1 (333-PPKPPR338; SEQ ID NO: 7) and Pro 2 (355-PPEPHPVPPILTPSPPSAFP-374; SEQ ID NO: 9) domains of PTP-PEST were deleted by PCR. Briefly, an anti-sense oligonucleotide upstream of the Pro 1 (5'-TCGCTCGAGAGAGTCTTGCTTCTC-3'; SEQ ID NO: 10) designed with a Xho I site was used to amplify the cDNA of PTP-PEST in combination with the sense oligonucleotide T7. This PCR product, encoding for the N-terminus of PTP-PEST, was gel purified (QIAEXII, Qiagen) and digested with Not I and Xho I. A second PCR product encoding for the C-terminus of PTP-PEST was generated using a sense oligonucleotide with an Xho I site designed downstream of the Pro 1 (5'-CCACTCGAGACTCGAACAGTTGCCTT-3'; SEQ ID NO: 11) and an anti-sense oligonucleotide with a Xba I site (5'-CCTCTAGATCATGTCCATTCTGAA-3'; SEQ ID NO. 12). The PCR product, encoding for the C-terminus of PTP-PEST, was gel purified and digested with Xho I and Xba I. To reconstitute the full length PTP-PEST with the deleted Pro 1 region, the digested PCR products encoding for the PTP-PEST N and C-terminus domains were ligated in the Not I and Xba I sites of pACTAG. The Pro 2 region of PTP-PEST was deleted using a similar strategy. The deletions were verified by dideoxy sequencing of the mutated region using Sequenase (Amersham). Truncations of the GST PTP-PEST 344-437 (Pro 2) (GST 344-427, 344-417, 344-407 and 344-397) were performed by PCR using T7 primer and PTP-PEST specific oligos harboring a EcoR I site. The PCR products were digested with BamHI and EcoRI and ligated in the BamHI and EcoRI sites of pGEX RC. GST PTP-PEST 344-385 was generated by digestion of the pGEX RC 344-437 vector with KpnI and EcoR I, treatment with Mung Bean nuclease followed by religation of the plasmid.
- The constructs encoding for the N-terminus, C-terminus, LIM1, LIM2, LIM3 and LIM4 of avian paxillin fused to GST in the pGEX 2T vector were previously described (19). The paxillin GST LIM 1-3 was constructed by subcloning from the GST C-terminus pGEX 2T construct the BamH I/Sac I fragment in pBluescript 11 (KS). The BamHI/SacI fragment was then subcloned from pBluescript 11 (KS) to pGEX RC in the BamH I/Sac I sites. The paxillin GST LIM 1-2 of paxillin was constructed by subcloning

- the BamH I/Xma I fragment from the GST C-terminus construct in pGEX RC in the BamHI/XmaI sites. The paxillin GST LIM 3-4 was constructed by subcloning the XmnI/EcoRI fragment from the GST C-terminus of paxillin in pGEX 2TK in the SmaI/EcoRI sites. The paxillin GST C-terminus Δ LIM 3 was constructed by digesting
5 the paxillin GST C-terminus construct with Xma I and Sac II, followed by treatment with Mung Bean nuclease and religation of the plasmid.
- Site directed mutagenesis** Eight proline residues (highlighted) found in the Pro 2 domain of PTP-PEST (355-PPEPHPVPPILTPSPPSAFP-374; SEQ ID NO: 9) were mutated to alanine residues by PCR. Briefly, mutagenic oligonucleotides were
10 engineered: (5'-CAGCCACCAGAAGCTCACCCGGTGC-3'), P358A (SEQ ID NO:13)
(5'-CCAGAACCTCACGCCGGTGCCACCCATC-3'), P360A (SEQ ID NO: 14)
(5'-CCTCACCCGGTGGCACCCATCCTGAC-3'), P362A (SEQ ID NO: 15)
(5'-CACCCGGTGCCAGCCATCCTGACGC-3'), P363A (SEQ ID NO: 16)
(5'-CCCATCCTGACGGCATCACCTCCTTC-3'), P367A (SEQ ID NO: 17)
15 (5'-CTGACGCCATCAGCTCCTCAGCC-3'), P369A (SEQ ID NO: 18)
(5'-ACGCCATCACCTGCTTCAGCCTTCC-3'), P370A (SEQ ID NO: 19)
(5'-CCTTCAGCCTTCGCAACCGTTACCAC-3') P374A (SEQ ID NO: 20).
- pGEX RC PTP-PEST Pro 2 (aa 344-437) was used as a templates for the PCR reactions. Each oligonucleotide was used in combination with a pGEX anti-sense
20 specific oligonucleotide to amplify a portion of the Pro 2 region. The eight different PCR products were gel purified. A second PCR product was generated using a pGEX specific sense oligo in combination with a PTP-PEST specific antisense primer (5'CCATGTGCAGCACTGGCTT-3'; SEQ ID NO: 21) and was recovered by gel purification. In order to reconstitute the full length Pro 2 region, each mutagenic PCR
25 products was incubated with the second PCR product and a strand overlap extension step was performed with Vent DNA polymerase (New England Biolabs) for 7 cycles using the following conditions: 94 °C for 30 sec, 50 °C for 30 sec and 72 °C for 30 sec. Each of the products from the strand overlap extension step was amplified by PCR by adding 100 pool of the pGEX sense and antisense primers and using the same
30 conditions for 30 cycles. Each of the PCR products was gel purified and digested with BamH I and EcoR I and ligated in the BamH I and EcoR I sites of pGEX RC. Following transformation in E. coli DH5alpha, mutants were screened by DNA sequencing. Using a similar approach the cysteine 523 of paxillin was mutated to a serine. The other mutants of paxillin were previously described (19).
35 **In vitro** translation pcDNA3 paxillin WT, Δ LIM3, C467A, C470A, C467/470A, Δ LIM4, C523S and pcDNA3 were digested with Xho I. The linearized plasmids were used as templates for *in vitro* transcription using T7 RNA polymerase (NEB). Two μ l of the transcription reactions were used to performed *in vitro* translation in the presence of 35 S-methionine and rabbit reticulocyte lysate (Promega). The *in vitro* translated

products were tested for binding to 1 µg of GST PTP-PEST 344-397 or GST alone.

In vitro binding assay using GST fusion proteins NIH 3T3, NIH 3T3 Src Y527F, pervanadate treated NIH 3T3 and HEK 293T cells were lysed in HNMETG buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 1% Triton X-100 and

- 5 10% glycerol) supplemented with Complete protease inhibitors (Boehringer Mannheim) and 1 mM Na₃VO₄, as previously described (10). The lysates were cleared of cellular debris by centrifugation at 16 000 x g in a 4 °C microcentrifuge. Protein concentrations were determined using the Bradford method (Bio-Rad) using bovine serum albumin as a standard. PTP-PEST, p130Cas and paxillin GST fusion proteins were expressed by induction for 2 h of exponentially growing bacterial cultures with 1 mM IPTG and fusion proteins were extracted and immobilized on Glutathione Sepharose beads according to the manufacturer's recommendations. Aliquot of 200-250 µg of cell lysates, precleared with 1 µg of GST alone immobilized on Glutathione Sepharose (Pharmacia), were incubated with each PTP-PEST GST fusion proteins in 1 ml of HNMETG for 90 minutes at 4 °C. 200 µg of 293-T extract was incubated with each p130Cas and paxillin GST fusion proteins in 1 ml of HNMETG for 90 minutes at 4 °C. Following the binding incubation, beads were washed three times in HNTG buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol) and boiled in SDS sample buffer to elute bound proteins. Following SDS-PAGE and transfer to PVDF membrane (Immobilion-P), bound proteins were detected by western blotting with the appropriate antibodies.

- 20 **Immunoprecipitations** Cell monolayers were lysed in 1 ml of immunoprecipitation buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM Na₃VO₄ and Complete protease inhibitors) directly on the tissue culture dishes on ice for 15 minutes. The lysates were cleared of cellular debris by centrifugation at 16 000 x g in a microcentrifuge. 200-500 µg aliquots of cell extracts were precleared with 20 µL of Protein A agarose (Gibco-BRL). The cleared extracts were incubated with 2 µL of monoclonal anti-paxillin antibody or 1 µL of the polyclonal antibody against avian paxillin for 90 minutes at 4 °C. Immune complexes were recovered by addition of 20 µL of Protein A agarose for 90 minutes at 4 °C. Immunoprecipitates were washed three times in immunoprecipitation buffer and boiled in SDS-PAGE sample buffer. Following SDS-PAGE and transfer to PVDF membranes, bound PTP-PEST was detected by western blotting using the anti-HA antibody 12CA5. Blots were stripped and reblotted with the anti-paxillin antibody to verify equal precipitations.

- 25 30 35 **Western blotting** Non-specific sites of blotted PVDF membranes were blocked in TBS-T (20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.1% Tween-20) containing 1% (w/v) bovine serum albumin and 1% (v/v) goat serum for 60 minutes at room temperature. Primary antibodies were diluted in blocking buffer and incubated with blots for 60 minutes at room temperature. The monoclonal antibody anti-paxillin was used at a

dilution of 1:10000. The monoclonal antibodies 12CA5 and 4G10 were used at 1:2000 dilution. The monoclonal antibodies anti-p130Cas, PY20 and PY99 were used at 1:1000 dilution. Blots were washed extensively with TBS-T before incubation with the secondary antibody. The secondary anti-mouse antibody conjugated to HRP was diluted 1:10000 in blocking buffer and incubated with blots for 60 minutes at room temperature. Following repeated washing in TBS-T, bound primary antibodies were detected using chemiluminescence(NEN Lifescience)

5 Results

Paxillin is associated with PTP-PEST in various mouse tissues It was recently 10 reported that PTP-PEST and paxillin associate in chicken embryo (CE) fibroblasts and Swiss 3T3 cells (30). To verify that PTP-PEST and paxillin were physically associated *in vivo*, co-immunoprecipitation experiments were performed in lysates of various mouse tissues. As seen in figure 15, paxillin coprecipitated with PTP-PEST in liver, brain, heart, lung, spleen and thymus lysates. The highest amounts of PTP-PEST 15 associated paxillin were found in lung, spleen and liver. No paxillin was detected in PTP-PEST immunoprecipitate from kidney lysate since PTP-PEST is expressed in low levels in kidney (31). In addition, paxillin was not found in pre-immune IP made from liver extracts demonstrating the specificity of the association. When the blot was reprobed with a PTP-PEST antibody, comparable amounts of PTP-PEST were found 20 in the precipitates except in the kidney where no signal was detected (data not shown). These results demonstrate that the PTP-PEST-paxillin association previously described in fibroblasts (30) also occurs *in vivo* in a majority of mouse tissues.

Paxillin associates *in vitro* with a fragment of PTP-PEST containing a proline 25 rich region. In order to define the region of PTP-PEST involved in the binding of paxillin, we have used the non-catalytic C-Terminus tail of PTP-PEST in addition to several deletion mutants of PTP-PEST expressed as GST fusion proteins (Figure 16a). The GST PTP-PEST fusion proteins (aa 276-775, 276-613, 276-567, 276-453, 276-437 or GST alone) bound to Glutathione Sepharose were incubated with 500 µg 30 of proteins extracted from NIH 3T3 cells. Following a binding incubation period, the beads were washed several times and bound proteins were separated by SDS-PAGE. Associated paxillin was detected by western blotting. As seen in figure 16b (top panel), paxillin was detected in every binding assay except with GST alone. Coomassie blue staining of the GST fusion proteins used for the binding assay (figure 16b, bottom panel) was used to demonstrate the integrity of the purified proteins. From these 35 results, the paxillin binding site lies between the amino acids 276-437 of PTP-PEST. The only characterized protein-binding domain within aa 276-437 of PTP-PEST is a proline rich region (Pro 1, 333-PPKPPR-338; SEQ ID NO: 7) that acts as binding site for the SH3 domain of p130Cas, Hef1, Sin and Grb2 (6,9,10). Also present in this PTP-PEST fragment is Pro 2 (355-PPEPHPVPPILTPSPPSAFP-374; SEQ ID NO: 9) which

is a 20 amino acids segment rich in proline residues that has no determined function. In order to delimit the minimal region required for binding to paxillin, a binding assay was thus performed using Pro 1, Pro 2 344-437, Pro 2 344-427, Pro 2 344-417, Pro 2 344-407, Pro 2 344-397 and Pro 2 344-385 of PTP-PEST expressed as GST fusion proteins (figure 16a). Paxillin was associated with the Pro 2 344-437, 344-427, 344-417, 344-407 and 344-397 PTP-PEST fusion proteins (Figure 16c, top panel). GST Pro 2 344-385, GST Pro 1, GST Pro 5 and GST alone failed to associate with paxillin as seen in figure 16c (top panel). These results suggest that amino acids 344-397 of mouse PTP-PEST are sufficient for its association with paxillin. This peptide has the sequence of SEQ ID NOs: 22 and 23 (mouse and human, respectively). Of course, this sufficient binding sequence is not necessarily the minimal binding sequence.

The Pro 2 region of PTP-PEST is required for paxillin binding *in vitro* and *in vivo*. The Pro 2 domain of PTP-PEST lies between aa 355-374. Since the smallest GST Pro 2 fusion protein used in figure 15c encodes for aa 344-397, a PTP-PEST mutant lacking Pro 2 was generated to rigorously demonstrate its role in paxillin binding. A binding assay was performed using two paxillin GST fusion proteins: Paxillin N (encoding for the LD motifs) and Paxillin C (encoding for the 4 LIM domains). These two proteins in addition to GST p130Cas SH3 and GST alone were purified and incubated with cell extracts of HEK 293T transfected either with WT or ΔPro 2 HA-PTP-PEST as described above. The bound PTP-PEST proteins were monitored by western blotting using the anti-HA antibody 12CA5. As shown in figure 17a (top panel), the GST Paxillin C and p130Cas SH3 were bound to WT HA PTP-PEST. The GST Paxillin N and GST alone failed to bind WT HA PTP-PEST. Five µg of HA WT PTP-PEST transfected HEK 293T extract were loaded to demonstrate the proper expression of the transfected construct. When the same binding assay was performed using ΔPro 2 HA PTP-PEST transfected cell extracts, GST paxillin C failed to bind ΔPro 2 PTP-PEST (Figure 17a, top panel). The SH3 domain of p130Cas was still capable of association with the ΔPro 2 mutant of PTP-PEST since it only requires an intact Pro 1 domain. ΔPro 2 PTP-PEST was expressed to similar levels to WT as seen in the TCL (5 µg of proteins) lanes. To verify the presence and integrity of the GST fusion proteins, the blot were stripped and probed with an anti-GST polyclonal antibody (Figure 17a, bottom panel). From these results, it is clear that the Pro 2 motif of PTP-PEST is implicated in paxillin binding. In addition, the LIM domains of paxillin rather than the LD motifs are implicated in the association with PTP-PEST.

The importance of the Pro 2 domain of PTP-PEST for paxillin binding was also confirmed *in vivo* in a co-immunoprecipitation experiment. HEK 293T cells were transfected with either: Mock (empty pACTAG), HA WT PTP-PEST, HA ΔPro 1 PTP-PEST, HA ΔPro 2 PTP-PEST. The cells were lysed 48 h after transfection and

the expression of the transfected constructs was monitored by western blotting of TCL (5 µg) using the anti HA antibody 12CA5 as seen in figure 17b (top panel). Endogenous paxillin was then immunoprecipitated with a monoclonal antibody. The presence of HA PTP-PEST was assayed by blotting against the HA epitope using the 5 12CA5 antibody. Both the WT and ΔPro 1 PTP-PEST were found in paxillin immunoprecipitates as seen in figure 17b (middle panel). In accordance with the result shown in figure 17a, PTP-PEST lacking a Pro 2 was not found in paxillin immunoprecipitates (figure 17b, middle panel). Equal precipitation of paxillin from each sample was verified by stripping the blot and reprobing with an anti-paxillin monoclonal 10 antibody (figure 17b, bottom panel).

Mutational analysis of the Pro 2 region reveals the importance of proline 362 for paxillin binding activity. The Pro 2 domain of PTP-PEST is defined by 20 amino acids half of which are proline residues (Figure 18a). Although three PxxP motifs are found in the Pro 2, none of them have the consensus sequence for SH3 binding sites 15 (Class 1-RxxPxxP and Class 2PxxPxR) or WW domains (PPxY) (32). Eight of the proline residues were individually mutated to alanine (Figure 17a) in the context of the GST Pro 2 344-437 construct (see figure 16) in order to get a better understanding of this novel paxillin binding domain. The WT GST Pro 2 as well as the eight proline to alanine mutants were purified from induced bacterial cultures and incubated with NIH 20 3T3 cell lysates. As seen in figure 18b, paxillin was bound to all fusion proteins used except to the P362A mutant. Equal amounts of GST fusion proteins as well as the integrity of the products are shown in figure 18c. From these results, we can conclude that the proline residue 362 is critical for binding to paxillin, and should be present in a minimal binding sequence.

25 **The LIM domains 3 and 4 of paxillin are required for PTP-PEST binding.** Having delimited the region on PTP-PEST important for binding to paxillin, we were next interested in mapping the segment of paxillin required for its association to PTP-PEST. A GST fusion protein encoding the four LIM domains of paxillin was demonstrated to have the PTP-PEST binding activity (Figure 16a). A panel of GST fusion proteins of 30 paxillin LIM domains was generated in order to investigate which LIM domain is important for PTP-PEST binding. A schematic representation of these constructs is shown in figure 19a. These fusion proteins were purified from induced bacterial cultures and incubated with HA-PTP-PEST expressing 293T cell lysates. Bound PTP-PEST was detected by western blotting using the anti HA antibody 12CA5. As 35 seen in figure 19b, the paxillin carboxyl terminus (Paxillin C, LIM1-4) was bound to PTP-PEST. The C-Terminus deletion mutants (LIM 1-3, LIM 1-4ΔLIM3 and LIM 1-2) as well as the LIM domains expressed separately failed to bind PTP-PEST. Interestingly, the LIM 3-4 construct was the only deletion construct retaining PTP-PEST binding activity (Figure 19b). GST alone used as a negative control was not

capable of precipitating PTP-PEST. To demonstrate proper expression of each construct as well as the integrity of the products, a coomassie blue stained gel representative of the GST fusion proteins used in the binding assay is shown in figure 19c.

- 5 It is possible that only one of the LIM domains (3 or 4) is interacting with PTP-PEST, but both may be required to have a biologically active conformation. This may be an important concern especially since the fusion proteins have been expressed in bacteria. In order to test this hypothesis, co-immunoprecipitation experiments were performed between PTP-PEST and full length paxillin WT, Δ LIM3 or Δ LIM4 mutants.
- 10 HEK 293T cells were co-transfected with HA PTP-PEST and either pcDNA3 (empty), pcDNA3 WT, Δ LIM 3 or Δ LIM4 avian paxillin. Five μ g aliquots of each sample was analyzed by western blotting to verify PTP-PEST expression (Figure 20c). Aliquots of each lysate were immunoprecipitated with an antibody specific for avian paxillin. The presence of PTP-PEST in the paxillin precipitates was investigated by western blotting
- 15 using the anti-HA antibody 12CA5. As seen in figure 20a, PTP-PEST was detected after precipitation of WT paxillin but not either of the LIM 3 or 4 deletion mutants. To verify equal precipitation of paxillin in each sample, the blot was stripped and reprobed with a monoclonal antibody against paxillin. As seen in figure 20b, equal amounts of paxillin were detected in all the samples except the negative control (pcDNA3, figure 20b).
- 20 Intact LIM3 and LIM 4 are required for binding to PTP-PEST In order to confirm that both LIM 3 and LIM 4 domains of paxillin participate in PTP-PEST binding, point mutations were introduced in these domains. Each LIM domain is composed of two zinc fingers stabilized by critical cysteine, histidine or aspartic acid residues (19). The C467A and the C470A mutants disrupts the first and the second zinc finger of the LIM 25 3 domain respectively and the C467/470A is a double mutant. The C523S disrupts the first zinc finger of LIM 4. The WT paxillin and all the mutants were in vitro translated in the presence of 35 S-methionine. These products were then incubated with GST PTP-PEST Pro 2 (344-397; SEQ ID NO: 22). As seen in figure 21 (top panel), only the 30 WT paxillin was able to interact with the PTP-PEST GST fusion proteins. As a control, GST alone did not interact with WT paxillin (last lane, top panel). In the bottom panel, 15% of the amount of each product used in the binding assays is shown to demonstrate the integrity of the products and also as a loading control. These data clearly demonstrate that the integrity of both LIM 3 and LIM 4 is critical for binding to 35 PTP-PEST.

Paxillin is not a substrate for PTP-PEST Since paxillin is a tyrosine phosphorylated protein, a substrate trapping approach (7,8,10) using C231S mutants of the catalytic domain of PTP-PEST, was used to investigate if paxillin is a physiological substrate for PTP-PEST. The substrate trapping mutants used were GST 1-453 C231S

(containing Pro 2) and GST 1-354 C231S (Pro 2 is deleted) PTP-PEST fusion proteins. Protein extracts were prepared from control NIH 3T3, NIH 3T3 stably expressing Src Y527F and pervanadate treated NIH 3T3 cells. These samples were incubated with either PTP-PEST C-Terminus (aa 276-775), PTP-PEST 1-453 WT, PTP-PEST 1-453
5 C231 S or PTP-PEST 1-354 C231S expressed as GST fusion proteins. Tyrosine phosphorylated proteins precipitating with PTP-PEST GST fusion proteins were visualized by immunoblotting with an antiphosphotyrosine antibody. As seen in figure 22a, in the Src Y527F and pervanadate (PV) treated cells, a protein of 130 kDa is the major tyrosine phosphorylated protein bound to the C231S mutants of PTP-PEST. This
10 protein is known to be p130Cas (8,10) and thus serves as a control to ensure that the trapping experiment worked, as seen by blotting with an anti p130Cas antibody in figure 22c. The 60 kDa band detected in the PTP-PEST 1-354 C231S lanes is the GST fusion protein cross reacting in a non-specific manner with the PY99 antiphosphotyrosine antibody. In NIH 3T3 and NIH 3T3 Src 527F cells, a 70 kDa
15 tyrosine phosphorylated protein was detected in the PTP-PEST C-Terminus, 1-453 WT and C231 S but not in the 1-354 C231 S. This p70 protein was identified as paxillin when the blot was reprobed with an antipaxillin monoclonal antibody (figure 22b). Importantly, no paxillin was detected in the GST PTP-PEST 1-354 C231S lanes, the only construct lacking the Pro 2, thus unambiguously showing that the PTP-PEST
20 catalytic domain is not interacting directly with tyrosine phosphorylated paxillin. This result clearly demonstrates that paxillin is not a substrate for the PTP-PEST catalytic domain in a substrate trapping assay. Interestingly, when 3T3 cells are treated with pervanadate, only a small amount of paxillin is detected (overexposure of the blot, data not shown) bound to the PTP-PEST fusion proteins having the Pro 2. This suggest that
25 hyperphosphorylation of paxillin on tyrosine residues could prevent binding to PTP-PEST. The integrity of each fusion protein used in the trapping assay was verified by Coomassie blue staining of the PVDF blots as seen in figure 22d.

Discussion

In a recent report, Shen et al. (30) demonstrated that FAK complexes with
30 proteins having protein tyrosine phosphatase activity (PTP). One of these PTPs, PTP-PEST, was found in FAK complexes via a direct association with paxillin (30). These findings are significant since they suggest a dynamic regulation of protein tyrosine phosphorylation in focal adhesions. In support of this hypothesis, fibroblasts lacking FAK (33) or PTP-PEST (14) exhibit migration defects and a higher number of
35 focal adhesions indicating that both tyrosine kinases and phosphatases have an active function in the assembly and disassembly of these adhesive cellular structures. We have analyzed the binding of PTP-PEST to paxillin in order to get a better understanding of the function of PTP-PEST in the regulation of focal adhesion turnover.

The co-precipitation of paxillin and PTP-PEST was reported in CE (Chicken Embryo) and Swiss 3T3 cultured fibroblasts (30). We demonstrate that the association between paxillin and PTP-PEST is physiologically relevant since both proteins co-precipitate from a variety of normal mouse tissues namely liver, brain, heart, lung, 5 spleen and thymus (figure 15). Paxillin was not detected in PTP-PEST precipitate from kidney since PTP-PEST is poorly expressed in kidney as previously shown (31).

In this study, we have identified the domains in PTP-PEST and paxillin that are responsible for their association. Our present data demonstrate that *in vitro*, a segment of PTP-PEST from aa 344-397 was sufficient for binding paxillin. *In vivo*, a proline rich motif on PTP-PEST, Pro 2 (355-PPEPHPVPPILTPSPPSAFP-374; SEQ ID NO: 9), is essential for binding to paxillin. In addition, intact LIM 3 and LIM 4 domains of paxillin are required for binding to PTP-PEST Furthermore, tyrosine phosphorylated paxillin was not able to complex with a substrate trapping mutant of PTP-PEST (C231S) lacking the Pro 2 demonstrating that it is not a physiological substrate of PTP-PEST 10 15 20 25 30 35 in this "substrate trapping" type of assay. The catalytic domain of PTP-PEST is flanked by a carboxyl terminal tail rich in protein binding motifs. In this respect, the SH3 domains of p130Cas, Hef 1, Sin/Efs (10,34), Grb2, v-src (6) and Csk (31) have been shown to directly associate with proline rich sequences in PTP-PEST. In addition, the coiled-coil domain of PSTPIP also associates with a non-classical polyproline rich domain of PTP-PEST (35) and the PTB domain of Shc was shown to bind a NPLH motif on PTP-PEST (5). The Pro 2 of PTP-PEST contains none of the consensus sequences that can act as ligands for either SH3 or WW domains. This suggests that the Pro 2 of PTP-PEST is a novel protein binding motif that can associate with LIM domains of at least paxillin. Interestingly, Pro 2 is conserved between human and mouse PTP-PEST but is not present on other member of the PTP-PEST family of enzymes (PEP, PTP-HSCF and PTP20). One concern is that the Pro 2 deletion, which prevents paxillin binding, might produce a misfolded PTP-PEST. However, this hypothesis is unlikely since p130Cas can still interact with PTP-PEST ΔPro 2 through an SH3-Pro 1 association (figure 17a). In addition, site directed mutagenesis analysis demonstrated that Proline 362 is critical for binding to paxillin whereas seven other proline mutants had little if no effect on paxillin binding. To gain a better understanding of the Pro 2, experiments involving more point mutations of residues surrounding Pro 362 and a NMR structure determination of the peptide bound to its paxillin partner are currently under investigations.

Paxillin is an adaptor protein composed entirely of protein binding modules including LD, LIM, SH2 binding sites and proline rich domains. Our results indicate that only LIM 3 and LIM 4 are essential for PTP-PEST binding activity. When expressed as GST fusion proteins, neither LIM 3 nor LIM 4 alone were able to negotiate binding with PTP-PEST whereas a construct containing both LIM 3 and LIM 4 domains

supports the association. It is possible that LIM 3 and LIM 4 do not fold properly when expressed in bacteria, however a recent report by Brown et al. (25) indicates that GST LIM 2 and LIM 3 alone associate with serine and threonine kinases suggesting that they are properly folded domains (25). We have also observed that *in vivo*, full length paxillin lacking either LIM3 or LIM 4 was unable to bind to PTP-PEST. In addition, we have clearly demonstrated that point mutation that disrupt the zinc fingers in either LIM 3 or LIM 4 of paxillin prevents binding to PTP-PEST. Together, these data point to LIM 3 and LIM 4 as critical domains for paxillin association with PTP-PEST.

LIM domains ligands are extremely varied (27,28). For example, some LIM domains have been shown to heterodimerize while others bind to structurally distinct protein motifs (27). Among others, it has been previously shown that the LIM 3 of Enigma associates with a tyrosine based motif (tyrosine tight turn) of the β chain of the Insulin receptor (29). Interestingly, the tyrosine tight turn of the Insulin receptor (GPLGPLYA) contains a PxxP motif and mutation of the two prolines to alanines abolishes binding to the LIM 3 of Enigma (29). Furthermore, when the LIM 3 of Enigma was used to screen a random peptide library with a fixed tyrosine (position 0), prolines at position -1 and +2 were favored. A consensus sequence for the preferred ligand of the LIM 3 of Enigma was determined: GPHydGPLHyd(Y/F)A (Hyd=hydrophobic residue). Our data demonstrate that the Pro 2 of PTP-PEST, 355PPEPHPVPPILTPSPPSAFP-374 (SEQ ID NO: 9), is the binding site for paxillin and that Proline 362 (in bold) is critical for the association. The consensus ligand sequence for the LIM 3 of Enigma is not found in PTP-PEST Pro 2. LIM 3 and LIM 4 of paxillin thus associates with a novel polyproline motif, and adds to the wide variety of LIM domain ligands. The discovery of other ligands for LIM 3 and LIM 4 of paxillin and their comparison to the PTP-PEST Pro 2 will allow the elaboration of a preferred ligand sequence for these LIM domains.

In parallel to this work, PTP-PEST has also been reported to associate with the paxillin homologue Hic-5 (36). The C-terminal LIM domains of Hic-5 is 68% similar to the LIM domains of paxillin. It was shown that the LIM 3 of Hic-5 is the most important for the binding to PTP-PEST but is still not sufficient. Surprisingly, LIM 4 was not critical for the binding of Hic-5 to PTP-PEST. In addition, point mutations in the zinc finger of either LIM 3 or LIM 4 of Hic-5 did not prevent association with PTP-PEST in a co-precipitation experiment. These results differ from the one observed in the association between PTP-PEST and paxillin, and suggest that even though the LIM domains of Hic-5 and paxillin are 68% similar, the mechanism of binding to PTP-PEST is not identical.

PTP-PEST has been shown to be very selective for its physiological substrates (8,10). The selectivity towards p130Cas can be explained by the fact that both a substrate recognition by the PTP domain and a SH3-mediated association occur

before dephosphorylation. An important issue that needed to be resolved in order to understand the significance of paxillin-PTP-PEST association was to clarify if paxillin is a substrate for PTP-PEST. Our data clearly demonstrates that tyrosine phosphorylated paxillin was not bound to a PTP-PEST C231S mutant lacking the Pro 5 2 indicating that paxillin is not directly recognized by the PTP domain. In addition, equal amounts of paxillin were also found in the precipitates of GST PTP-PEST WT or C231S and paxillin was not found to be more tyrosine phosphorylated in the C231S samples suggesting an absence of cooperation between the catalytic domain and the Pro 10 2 (figure 22c). In support of these findings, Garton and Tonks (16) have demonstrated that overexpression of PTP-PEST in Rat-1 fibroblasts prevents cells to migrate in a wound healing assay. In these cells, p130Cas phosphotyrosine level was greatly reduced whereas paxillin and FAK tyrosine phosphorylation levels were unaltered (16). Thus, these results also support that paxillin is not a target for PTP-PEST.

Other PTPs were reported to have remarkable specificity towards substrates including PTP1B (7,37), T cell-PTP (38) and SHP-1 (39). In contrast, the presence of the PSTPIP binding motif on PTP-HSCF was demonstrated to be essential for a specific tyrosine dephosphorylation of PSTPIP since the PTP domain alone did not dephosphorylate PST-PIP (35). Because we based our conclusions only on a substrate trapping approach, it remains a possibility that paxillin is a weak substrate for PTP-PEST *in vivo*. It is also possible that the formation of some protein complexes could favor paxillin dephosphorylation by PTP-PEST. In a *in vitro* dephosphorylation assay, GST-PTP-PEST dephosphorylated weakly a paxillin peptide compare to a p130Cas peptide. The known promiscuous activity of PTPases *in vitro* prevents us to base our substrates identification using such an assay.

If paxillin is not a substrate for PTP-PEST, what is the physiological significance of paxillin-PTP-PEST association? A first clue to answer this question came from findings by Brown et al. (19) indicating that the intracellular localization of paxillin depends on the association of a yet unknown binding protein to the LIM 3 of paxillin.

30 A reasonable assumption is that this LIM 3 ligand must co-localize with paxillin in focal contact sites. PTP-PEST is most likely not the protein responsible for paxillin focal adhesion localization since it is found mainly in the cytoplasm. We have demonstrated in a previous study that PTP-PEST can translocate to the membrane periphery following integrin engagement (14). Hence, we propose a model where PTP-PEST is

35 translocated (14) to focal adhesions and associates with paxillin (figure 9a). This would allow the SH3-mediated association with focal adhesion located p130Cas and inhibit its downstream signalling via dephosphorylation of residues of p130Cas critical tyrosine residues. Importantly, the LIM 3 of paxillin would be negotiating to bind with the Pro 2 of PTP-PEST instead of associating with its ligand that is essential for focal

adhesion targeting (19). The SH3 domain of p130Cas has also been shown to be critical for proper focal adhesion targeting of p130Cas (40) probably via its association with p125FAK. In a similar manner to the LIM 3 of paxillin, the SH3 domain of p130Cas would be bound to the Pro 1 of PTP-PEST instead of p125FAK. Together, this 5 cascade would result in the release of paxillin and p130Cas from focal adhesion complexes in addition to p130Cas tyrosine dephosphorylation as seen in figure 9b. The additional recruitment of Csk via a direct association to PTP-PEST (31) would result in the phosphorylation of the inhibitory site of Src thus inhibiting the formation of new focal adhesions. Our findings that PTP-PEST knock-out cells accumulate large 10 numbers of focal adhesions suggest that the most important function for PTP-pest is to promote focal adhesion disassembly, hence the turnover of focal adhesion complexes (14). Therefore, we propose that PTP-PEST promotes focal adhesion turnover via its tyrosine phosphatase activity towards p130Cas and via direct binding to critical domains of p130Cas and paxillin required for focal adhesion targeting.

15 **EXAMPLE 5**

THERAPEUTIC UTILITY OF PTP-PEST INHIBITORS:

From the foregoing results, it is expectable that any agent capable of interfering with the binding of PTP-PEST with domains of signalling proteins would interfere with cell migration and/or cellular division. Such an effect would have a desirable clinical 20 utility in the treatment of tumors, in the prevention of pro-inflammatory cell recruitment as well as in the prevention of undesirable angiogenesis. These agents are considered specific to PEST and include specific inhibitors, competitive binding peptides, monoclonal antibodies to the binding sites of the substrate or of the enzyme or to the catalytic site of the enzyme.

25 As such preferred agents, two peptides have been designed to compete with the native enzyme for its natural substrates, namely p130Cas, through their SH3 domain:

Peptide 1: Thr Thr Gly Thr Met Val Ser Ser Ile Asp Ser Glu Lys Gln Asp Ser Pro Pro
Pro Lys Pro Pro Arg Thr Arg Ser Cys Leu Val Glu Gly (SEQ ID NO: 4), or a shorter 30 sequence such as Peptide 2: Gln Asp Ser Pro Pro Lys Pro Pro Arg Thr Arg (SEQ ID NO: 24)

Furthermore, peptides may be designed to interfere with the binding of PEST with signalling proteins involved in cell migration and/or proliferation, even if the latter are not substrates to PEST. Such an example of peptide has been given with the 35 peptide 344-397 (SEQ ID NO: 22) of PEST or a shorter sequence, which binds by its Pro 2 domain to paxillin. This binding peptide would expectedly compete with the native enzyme for the corresponding binding domain of paxillin.

The anti-tumoral activity may be achieved by interfering with a plurality of cellular events such as cellular division, tumor cell migration and endothelial cell

migration. As a result, the tumors cannot become invasive to the whole organism, they cannot divide, they are deprived from blood and oxygen nutrients, and they cannot get rid of metabolic wastes.

The peptides of this invention enter the target cells as verified with Pro 2 peptides in the example related to the binding of PEST to paxillin.

In that case, (data not shown) approximately 10 μ moles of Pro 2-TAT-HA fusion peptides successfully entered the cells and located in coronal focal adhesions, as seen by immunofluorescence with a mouse anti-HA Mab and a goat anti-mouse fluorescein conjugated secondary antibody.

The association of PTP-PEST and its binding partners has been broken *in vitro* by the addition of 500 nM of peptides (5). Considering that the successful usage of the TAT-fusion protein system of S. Dowdy requires as little as 15 nM (74), we trust that the TAT-Pro1 to TAT-Pro5 would become a method of choice to inhibit the activity of the PTP-PEST enzyme, and its subsequent effects in cell migration, invasion, angiogenesis and tumorigenicity. The active peptidic agents would be dosed to achieve an extracellular concentration of 10 nM to 1 mM. Preferably, an extracellular concentration of 0.1 μ M to 100 μ M would be formulated in therapeutic compositions, to provide intracellular concentration in the order of nanomolar concentration.

The compositions would further comprise a pharmaceutically acceptable carrier. Ideally, the above inhibitory peptides can be administered in any suitable forms and by any suitable route to deliver the proper amount thereof at the target tissue. Such carriers may comprise liposomes, immunoliposomes, nanoerythrosomes and derivatives thereof (such as PEG, immuno-nanoerythrosomes, etc.), nanoparticles and derivatives thereof (such as PEG particles, immuno-nanoparticles, etc.). The above peptides may also be modified to provide derivatives thereof. The term "derivatives" is intended to cover any peptidic variations comprising amino acid substitutions by natural or non-natural amino acid, additions of substituents on natural amino acid atoms, for the purpose of improving the pharmacological pharmacokinetics and pharmacodynamics of the peptides. The derivatives therefore provide compounds which comply with pharmacological requirements such as resistance to metabolic degradation, solubility, potency, etc.

As an alternative to the above peptides, antisense oligonucleotides to PEST can be made. The antisense molecules may be entrapped in the same pharmaceutical vehicles mentioned above for the peptides, and they may be modified to resist metabolic degradation, to be stable and/or to be attached to radioactive molecules by known techniques. The peptides and antisense DNA molecules may also be administered through plasmidic or viral vectors. The plasmid or vector may express or not the peptide. If the vector is expressed, the peptide acts as a competitor to the native PTP-PEST enzyme. Alternatively, the antisense mRNA molecules transcribed

from the plasmid or vector may combine to the endogenous messenger RNA molecules encoding the native enzyme. In the latter case, the expression of the native enzyme is decreased or shut down. To ensure proper targeting of the tissue to be treated, the antisense molecules or peptides may be coupled to a ligand capable of specifically and selectively binding a target cell receptor.

5

EXAMPLE 6**DESIGN OF PEPTIDES INTERFERING WITH THE BINDING OF A PHOSPHATASE TO A SIGNALLING PROTEIN IS DERIVED FROM BINDING STUDIES**

The binding studies between p130Cas and PEST, and between paxillin and PEST provide valuable information on the nature of the peptides potentially clinically useful.

Although all the agents named above are directed to PEST, other agents directed to the SH3 domains or other domains important in the activity of signalling proteins, may envisageably be made. These agents may also be derived from the same binding studies.

As compiled in the Table below, the skilled reader will appreciate that the same binding studies may be reiterated with a panoply of different PEST proline-rich regions and of binding signalling proteins, and this for the purpose of treating different pathologies. Some of signalling proteins are ubiquitous, other are more tissue-specific. Tissue-targeting capacity of the pharmaceutical compositions may be improved via the carrier and specific diseased tissue treatment is contemplated.

	Domains	Amino acids	Ligands	Expression patterns	References
25	PTP Pro 1	71-312 333-338	p130Cas p130Cas Hef1/Cas-L	Ubiquitous Ubiquitous Hematopoietic and epithelial cells	6 7,11 7
			Efs/Sin	Hematopoietic cells, placenta and brain	7
30	Pro 2	355-374	Grb2 Paxillin Hic-5	Ubiquitous Ubiquitous	4 This invention 36
	Pro 3		None identified	Ubiquitous	—
35	SHC binding Pro 4	599-602 675-680	SHC	Ubiquitous	5
	Pro 5	764-771	Grb2 Csk PSTPIP	Ubiquitous Ubiquitous	4 31 16
			PSTPIP2	Ubiquitous, higher in hematopoietic cells	
				Ubiquitous	52

40 While this invention has been described in connection with specific embodiments thereof, it will be understood that modifications can be made thereto

without departing from the spirit and teachings of this invention. These modifications are considered to be within the scope of this invention, as defined in the appended claims.

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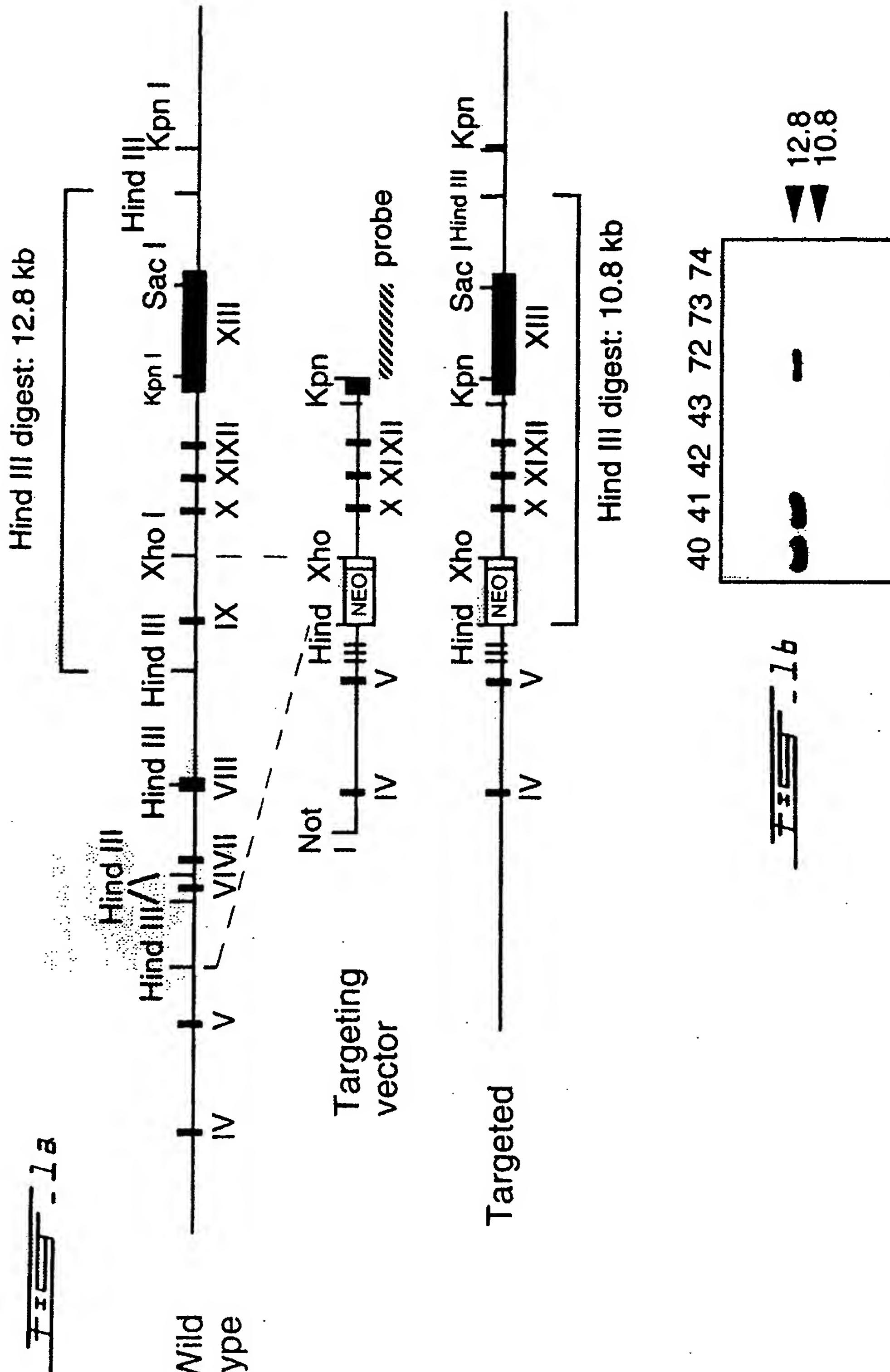
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What is claimed is:

1. A compound capable of interfering with the binding of phosphotyrosine phosphatase PEST to signalling molecules involved in cell migration, cell adhesion or cell division.
 - 5 2. A compound as defined in claim 1, wherein said signalling molecule is p130cas.
 3. A compound as defined in claim 1, wherein said signalling molecule is paxillin.
 4. A compound as defined in claim 2, which is a peptide comprising the amino acid residues 333 - 338 of SEQ ID NO. 1 or the amino acid residues 334-339 of SEQ ID NO. 2 .
 - 10 5. A compound as defined in claim 4 comprising the sequence defined in SEQ ID NO: 4 or 5 or a derivative or a part thereof capable of binding to p130cas.
 6. A compound as defined in claim 3, which is a peptide comprising the amino acid residues 355 - 363 of SEQ ID NO. 1 or the amino acid residues 356 - 364 of SEQ ID NO. 2 .
 - 15 7. A compound as defined in claim 6 comprising the sequence defined in SEQ ID NO: 22 or 23 or a derivative or a part thereof capable of binding to paxillin.
 8. A pharmaceutical composition comprising an anti-proliferative or anti-cell adhesion or anti-migrating amount of a compound as defined in any one of claims 1 to 7 and a pharmaceutically acceptable carrier.
 - 20 9. A pharmaceutical composition as defined in claim 8, which is dosed so as to provide an extracellular concentration of said compound comprised between about 10nM to 1 mM.
 10. The use of a compound as defined in any one of claims 1 to 7 in the making of a medication for treating a disease which has an etiological component selected from cell proliferation, cell migration, inflammation and angiogenesis.
 - 25 11. The use defined in claim 10 wherein said disease is cancer.
 12. A method for finding a genuine substrate for an enzyme in a cell that normally expresses the wild-type form of said enzyme, said method comprising the steps of:
 - modifying said cell to silence the expression of said enzyme, which silence has a detectable differential effect on said genuine substrate compared to the substrate of the cell which expresses the wild-type enzyme, whereby a silencious cell line is obtained;
 - providing a construct comprising a mutant form of said enzyme, said mutant being inactive toward said substrate but capable of binding thereto;
 - contacting the cell contents of said silencious cell line with said construct of said mutant, in order to obtain bound complexes of said substrate and said mutant; and
 - detecting said substrate.
 - 30 13. A method as defined in claim 12, wherein said silencious cell line is a null cell line for said enzyme.

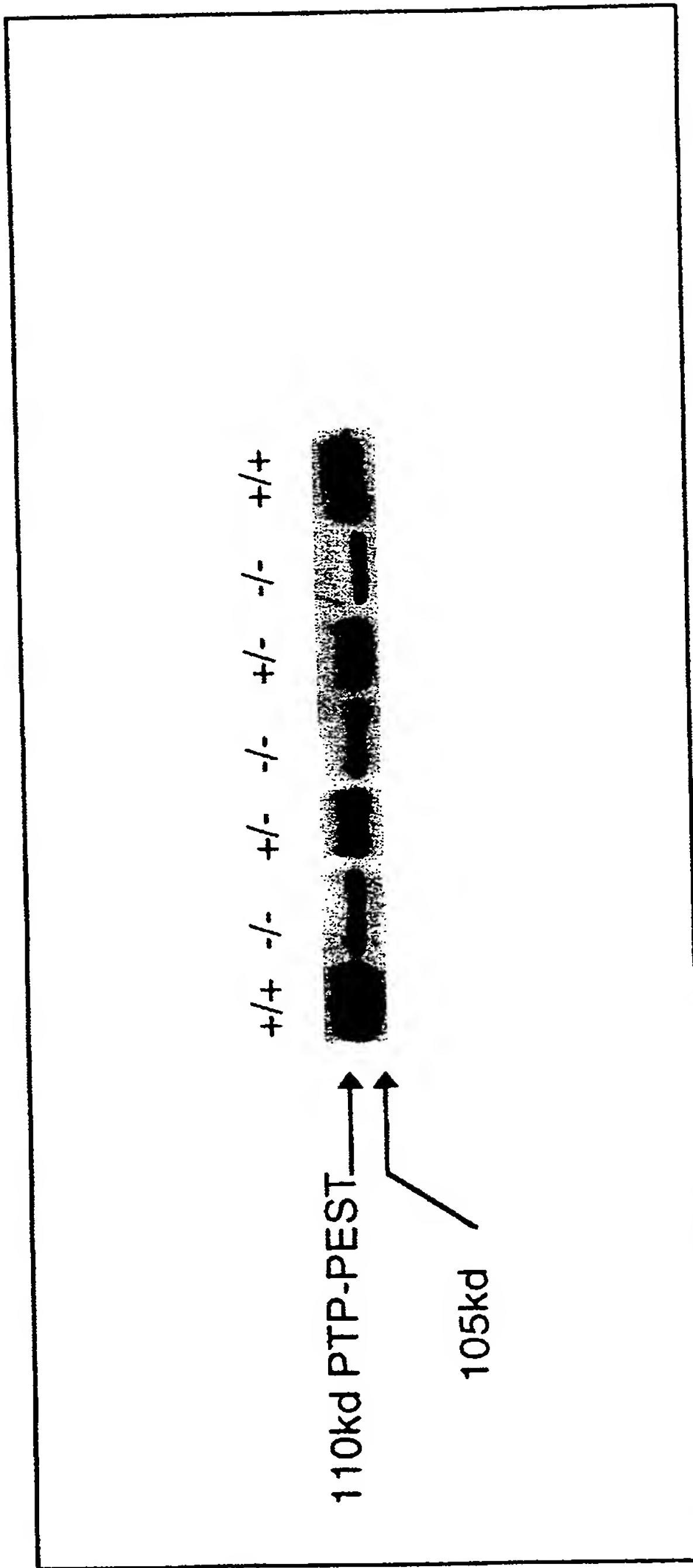
14. A method as defined in claim 12 or 13, wherein said enzyme is a protein phosphatase.
15. A method as defined in claim 14, wherein said protein phosphatase is a protein tyrosine phosphatase.
- 5 16. A method as defined in claim 15, wherein said protein tyrosine phosphatase is PTP-PEST.
17. A method as defined in claim 16, wherein said mutant form of PTP-PEST is conferred by the replacement of a cysteine residue at position 231 of SEQ ID NO: 1 or of SEQ ID NO: 2 by a serine residue.
- 10 18. A method as defined in any one of claims 14 to 17, wherein said differential effect on said substrate is a hyperphosphorylation effect.
19. A method as defined in claim 18, wherein said detecting step is performed with a ligand specific to a hyperphosphorylated substrate.
20. A method as defined in claim 19, wherein said ligand is an antibody.
- 15 21. A method as defined in any one of claims 16 to 20, wherein the presence of a hyperphosphorylated p130cas substrate is detected as a positive control of a genuine substrate.
22. A method as defined in any one of claims 12 to 21, wherein said mutant construct is bound to a solid support.
- 20 23. A method as defined in any one of claims 12 to 22, wherein said substrate is detached from said bound complexes prior to said detecting step.

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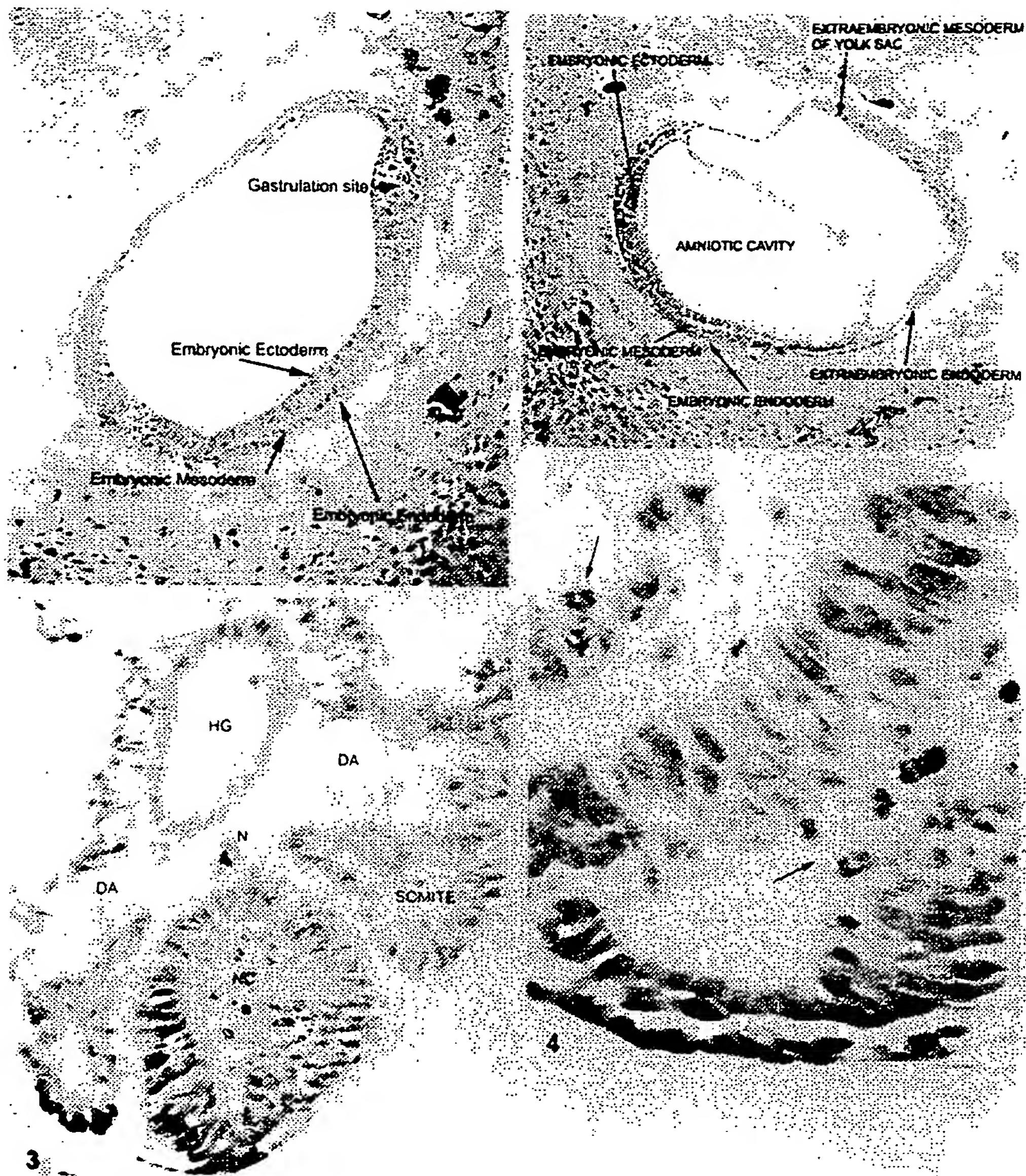
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Western blotting from PTP-PEST embryos.



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~~FIG. 2~~

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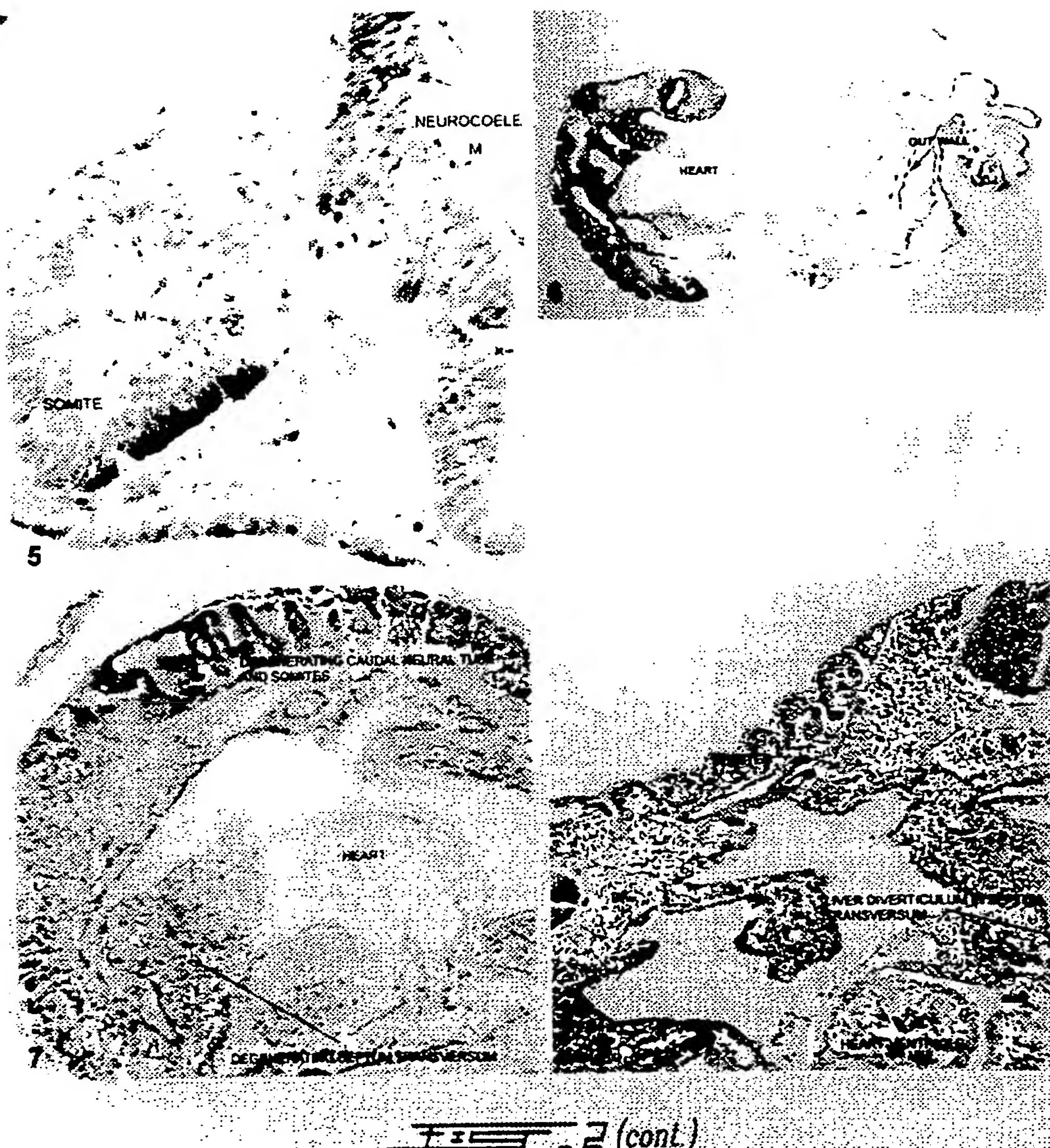


Fig 2 (cont)

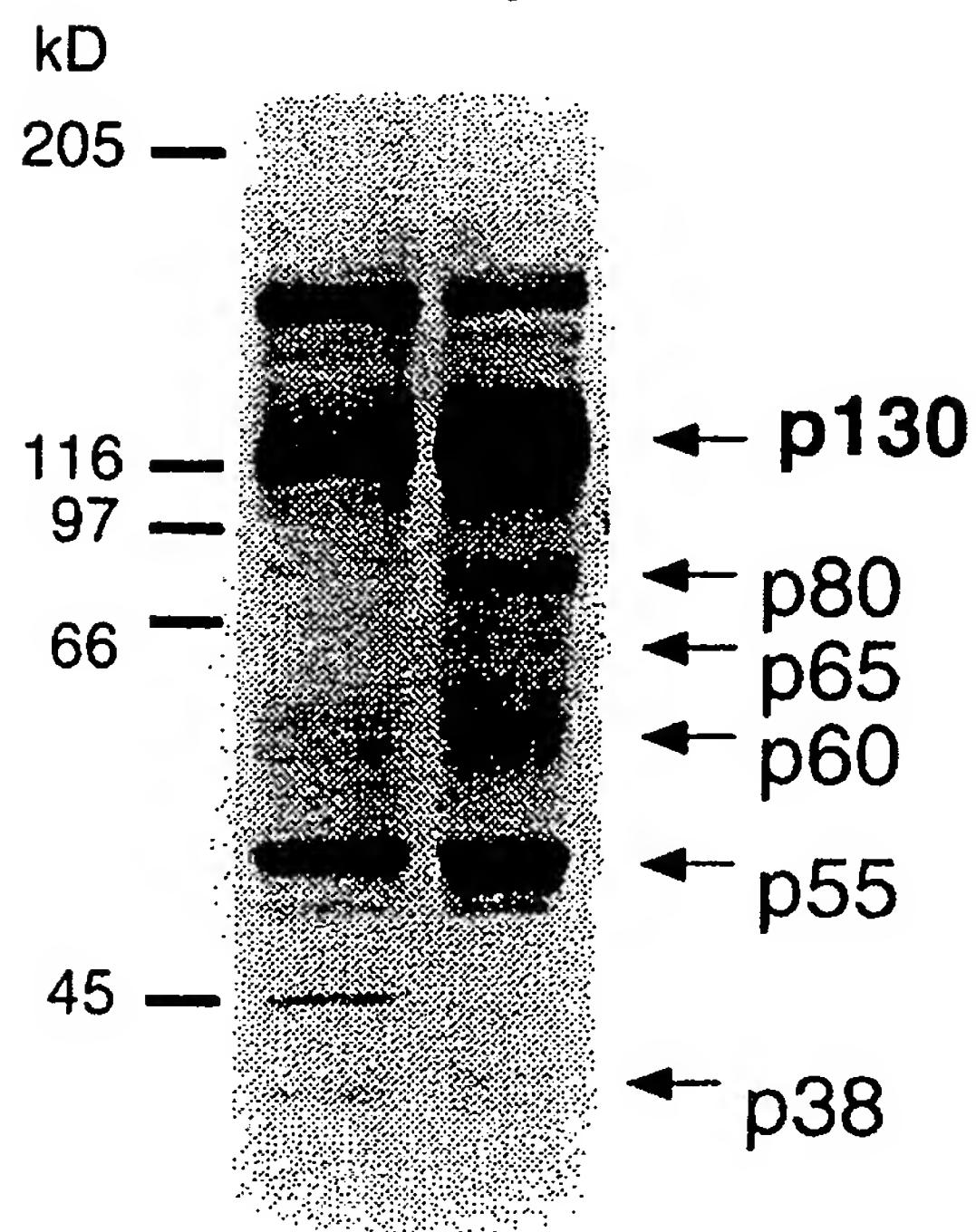
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~~Fig 3~~ Anti-pY western Blot
PTP-PEST K.O.

9 days p.c. embryos

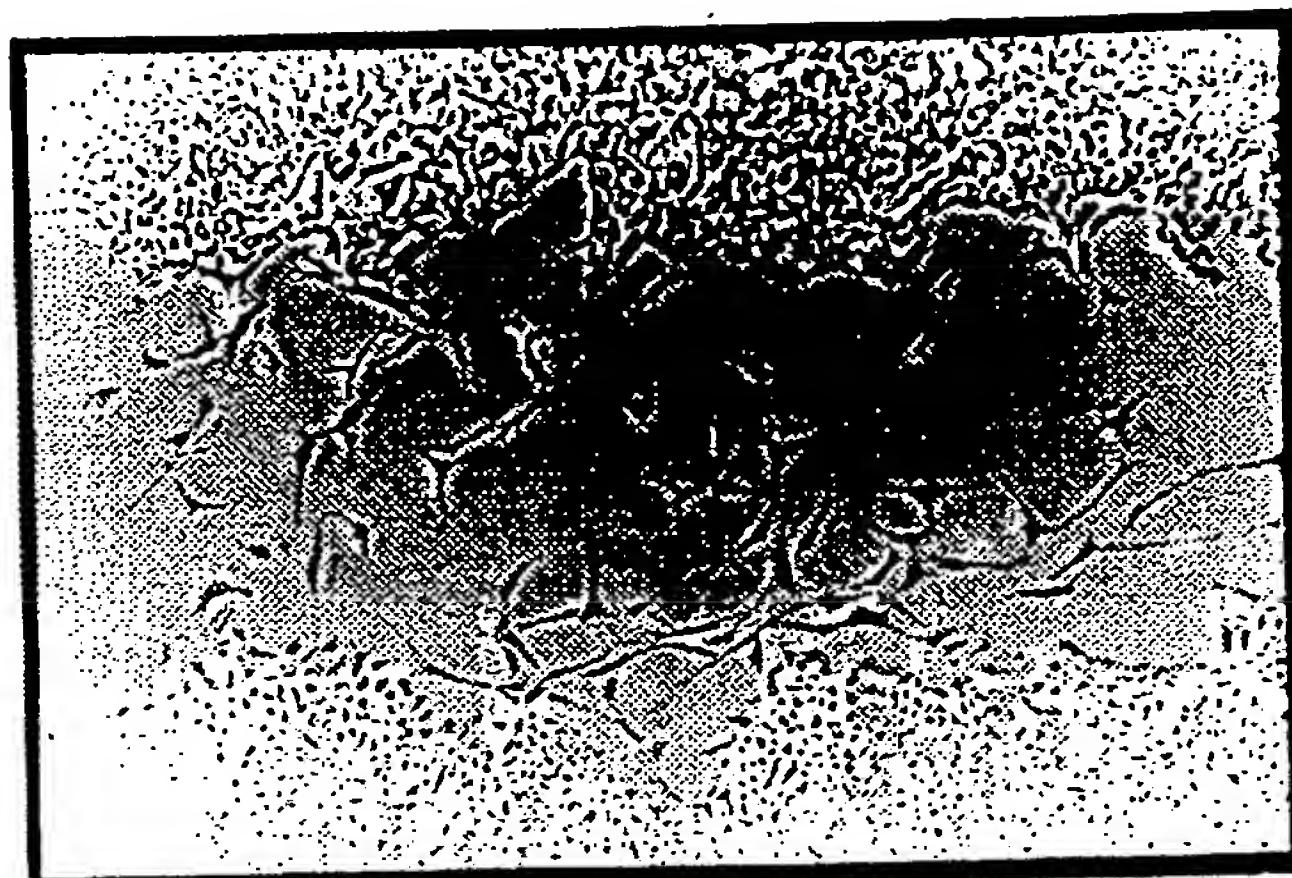
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+/-



-/-

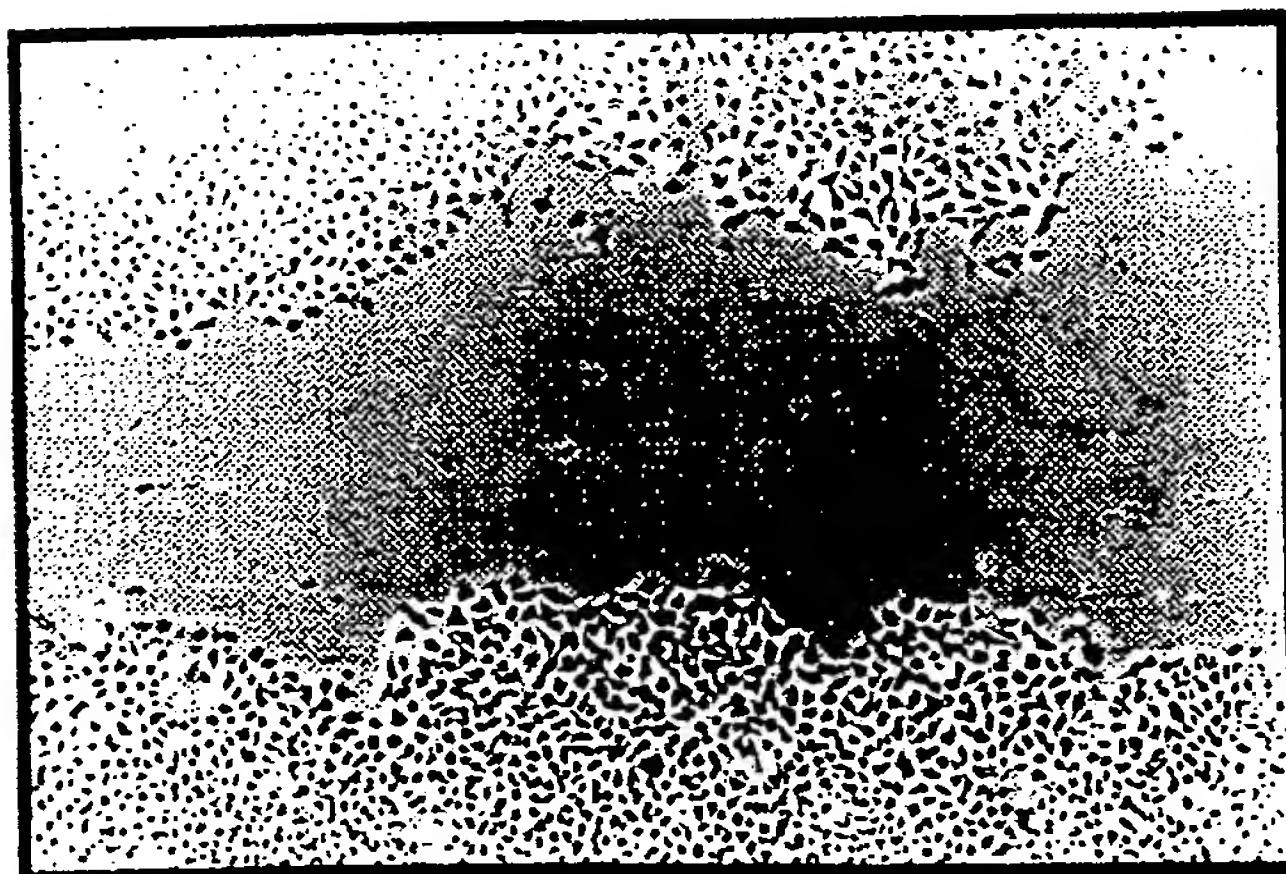


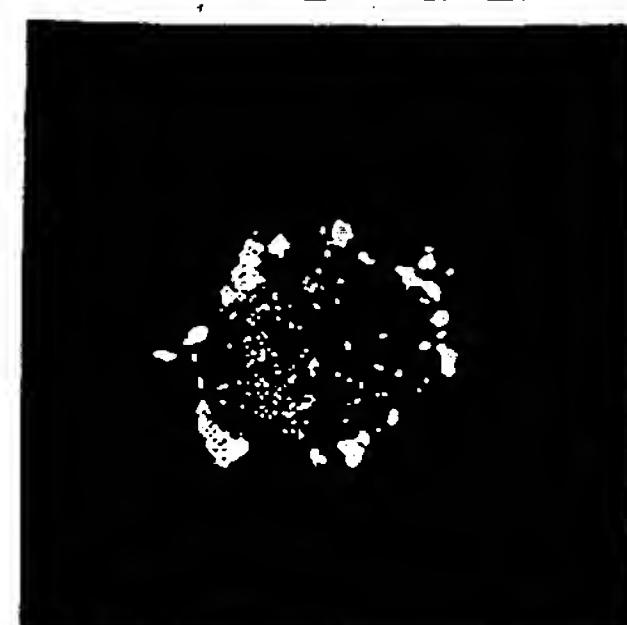
FIGURE - 4

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FIG - 5a

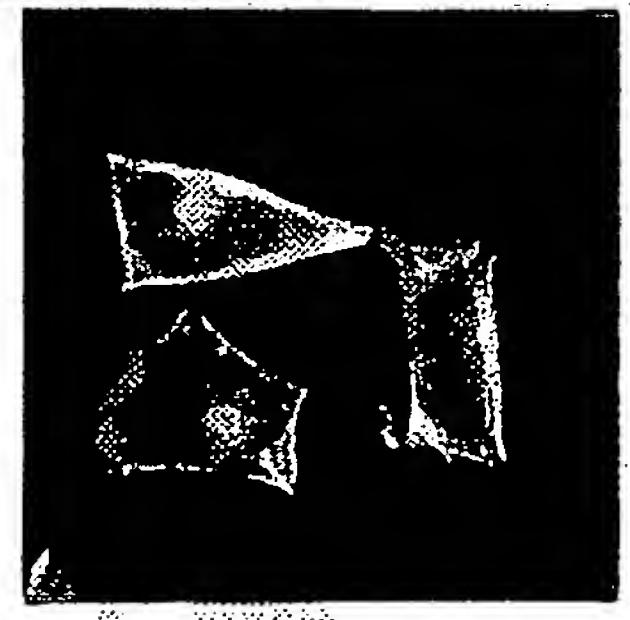
+/-

FIG - 5bFIG - 5c

-/-

FIG - 5dFIG - 5e

+/-

FIG - 5fFIG - 5g

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FIG - 5h

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Anti-
Phosphotyrosine

Loading
Control

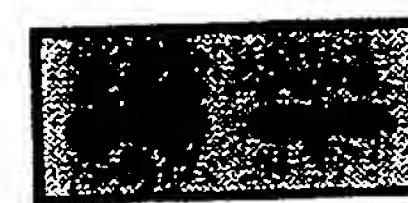
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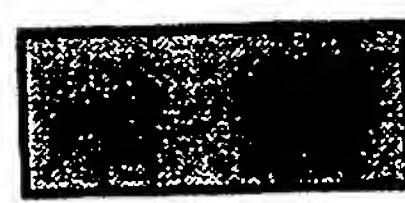
Cortactin



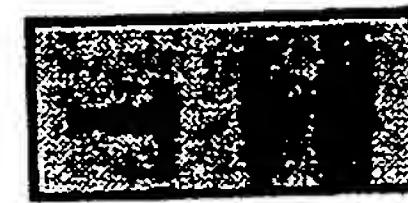
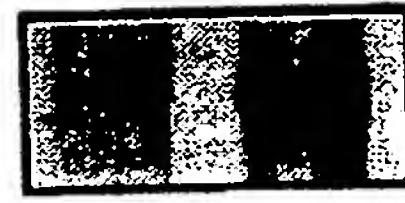
FAK



Paxillin

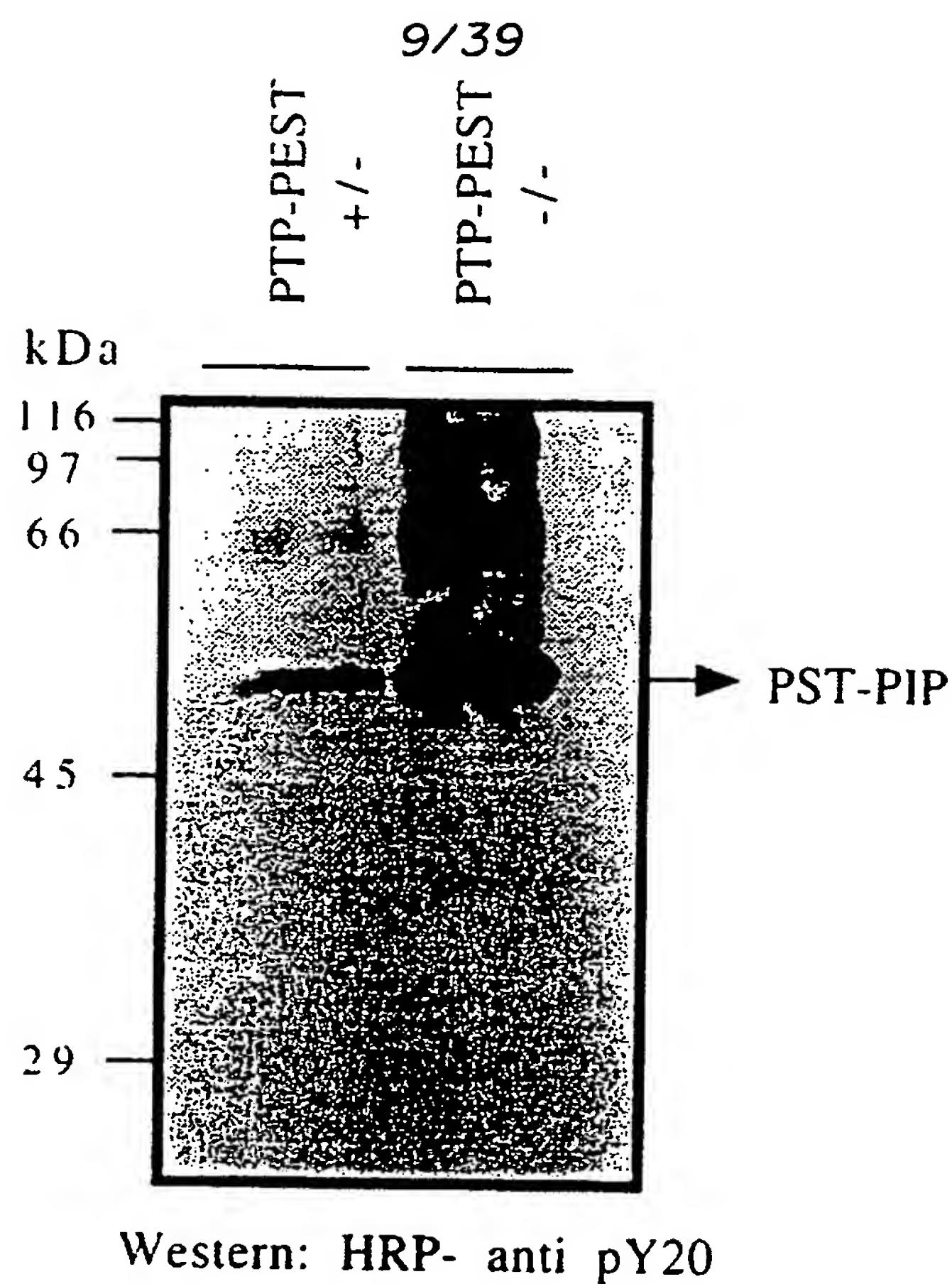


p130cas



FEB-6

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Western: HRP-anti PST-PIP

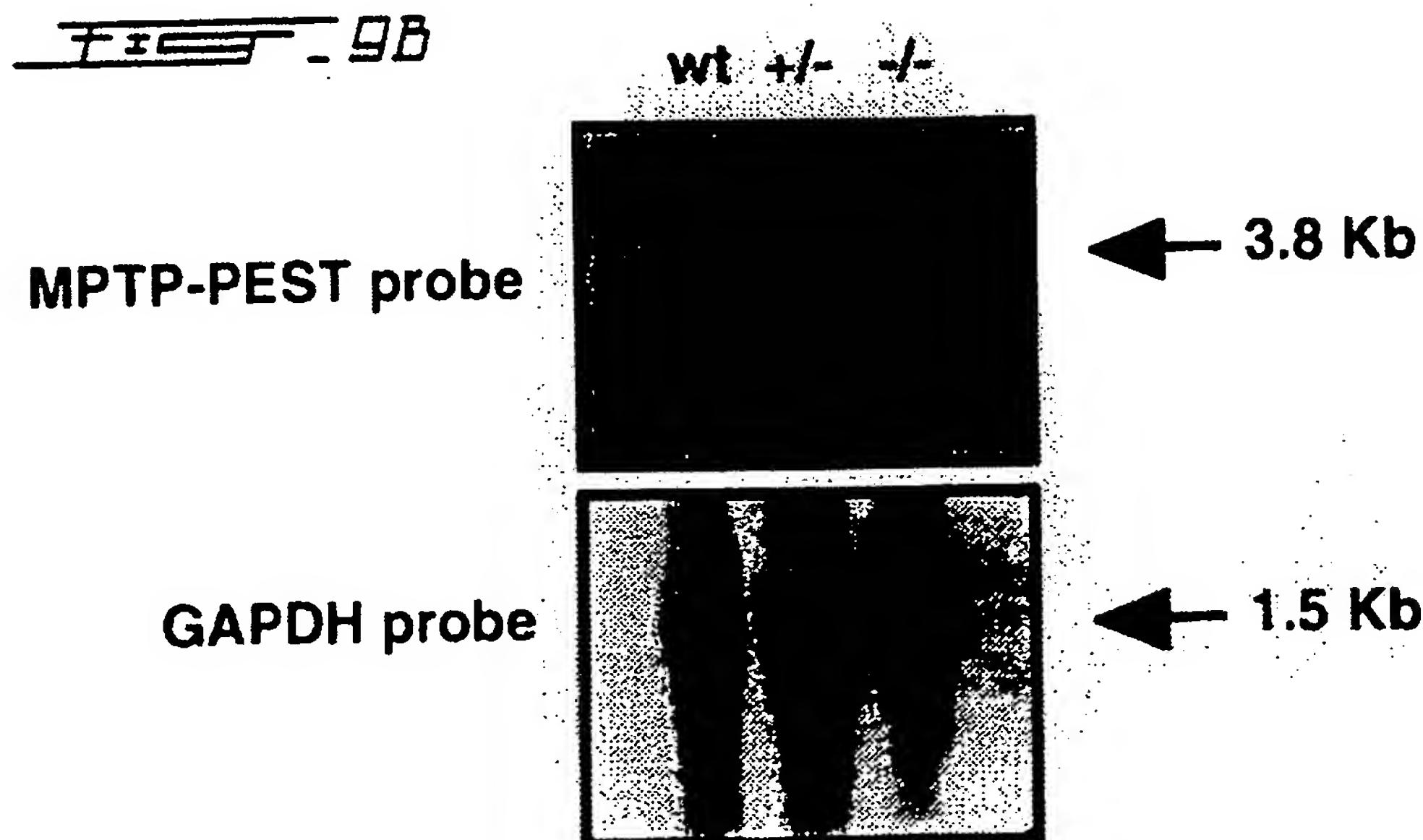
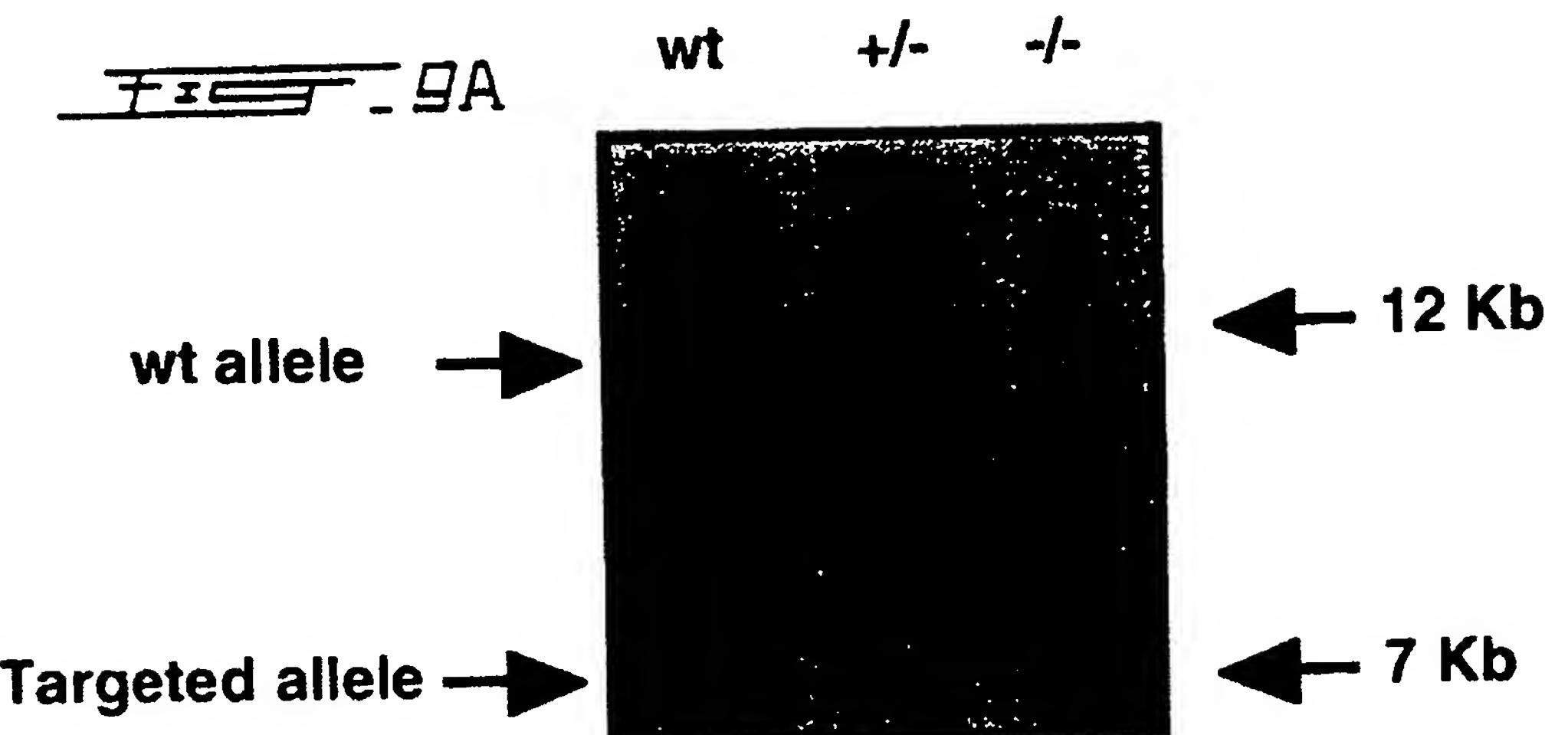
F x — 7



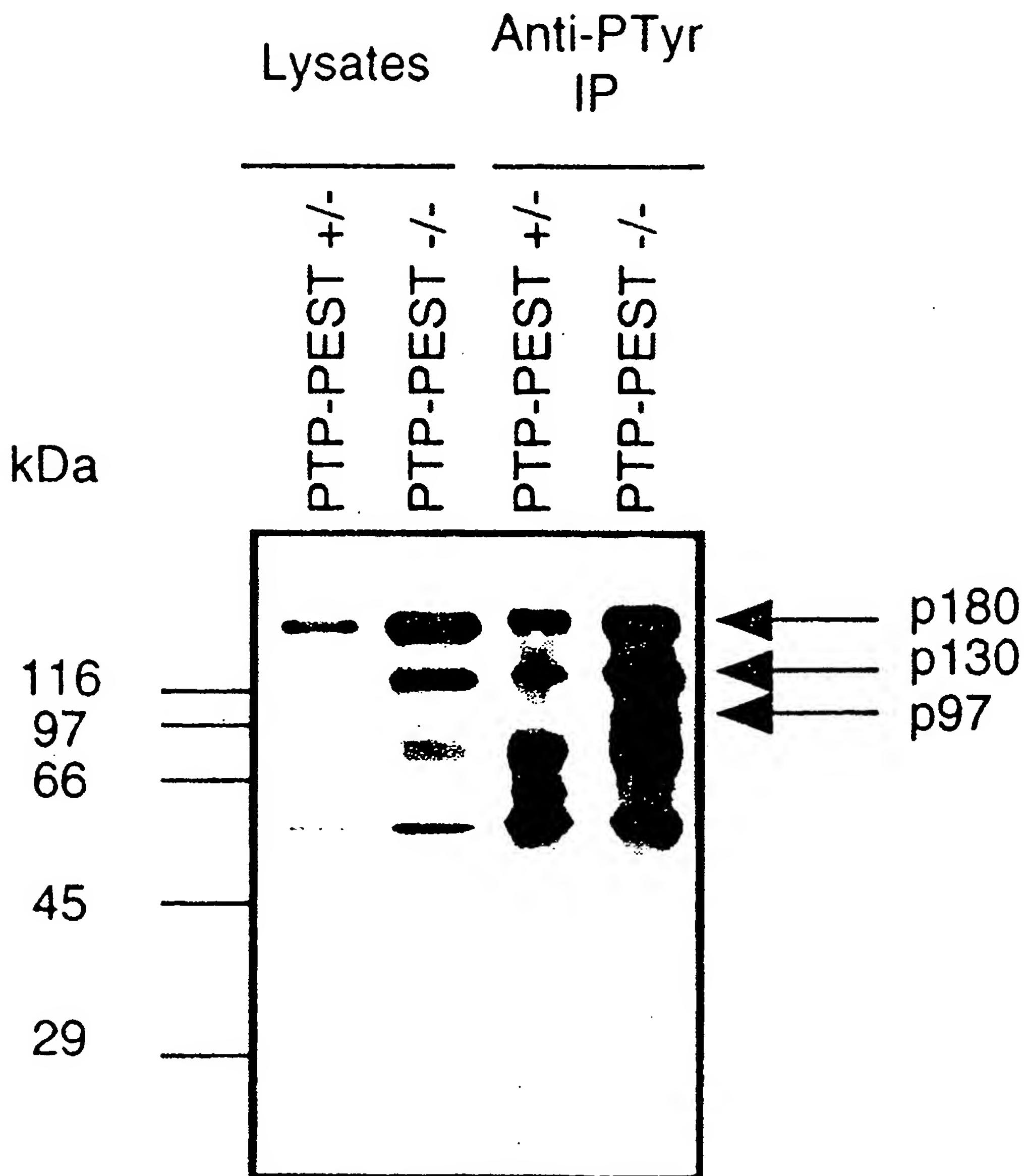
F x — 8

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Western: Antiphosphotyrosine

王二 - 10A

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12/39
130Cas
IP

kDa

205

116

97

66

Western: anti-pTyr

116

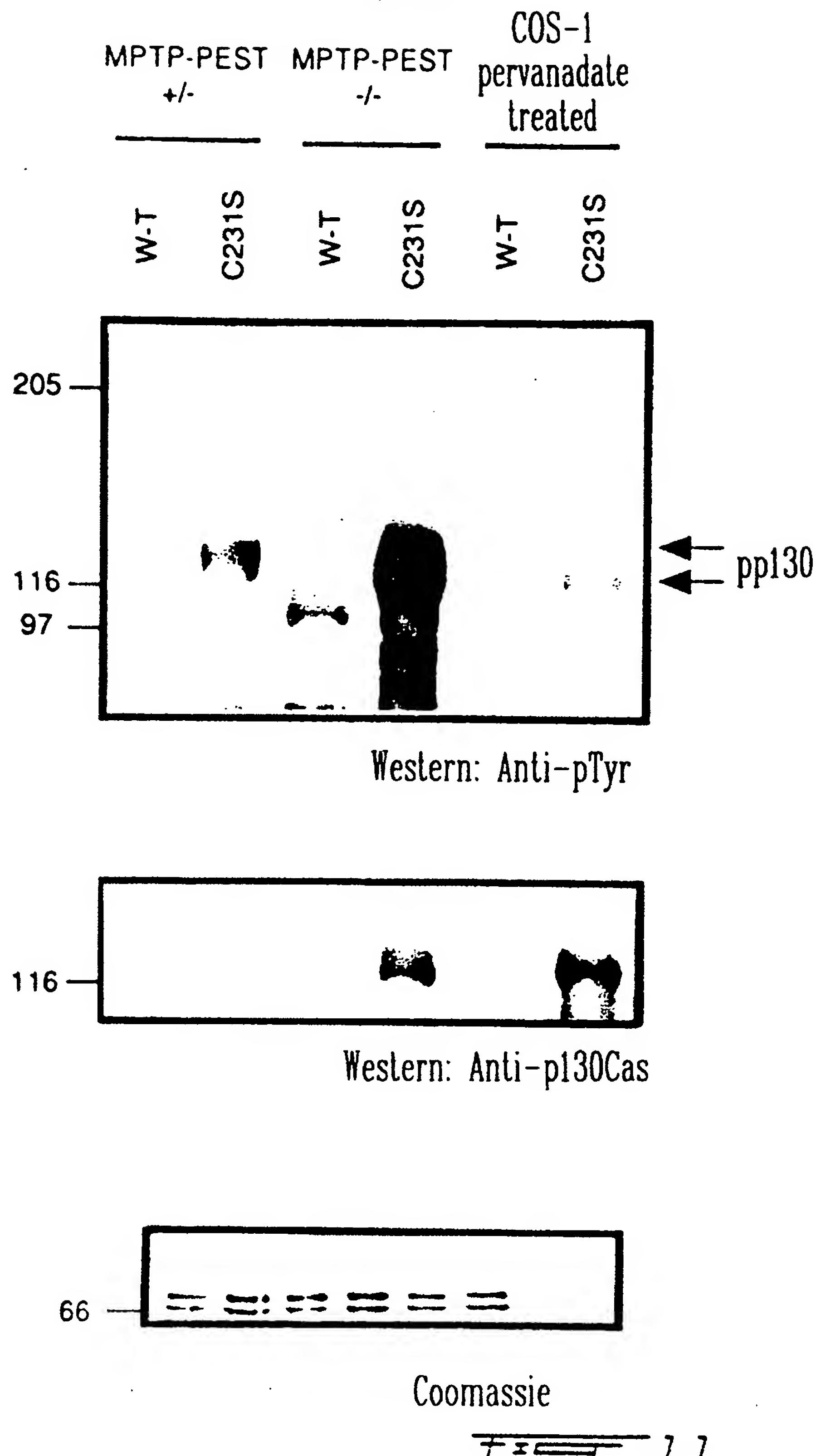
A black and white photograph showing two dark, irregular shapes, possibly artifacts or noise, against a white background. A horizontal line from the number 116 points to the left edge of the frame.

Western: anti-p130Cas

Test - 10B

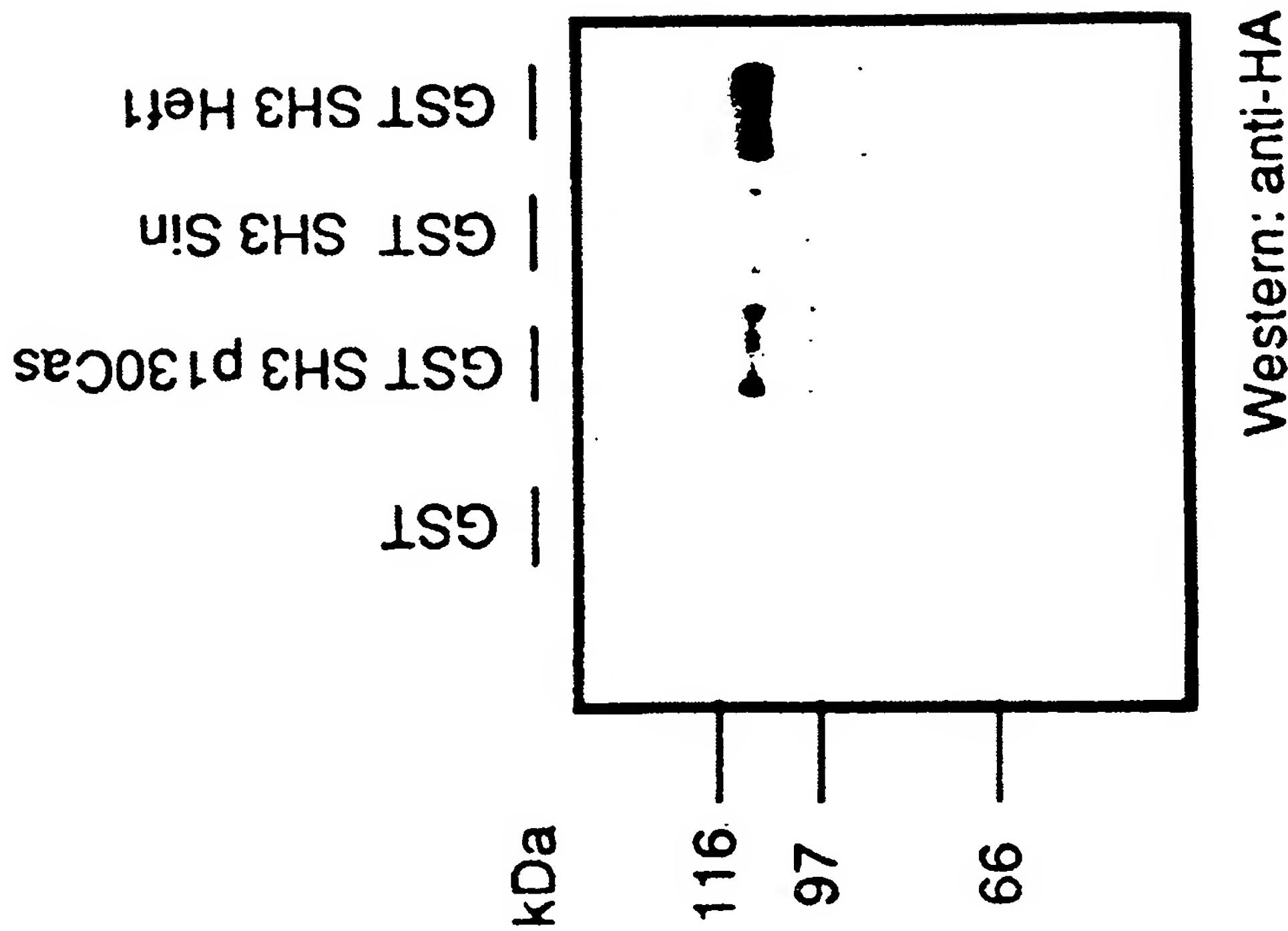
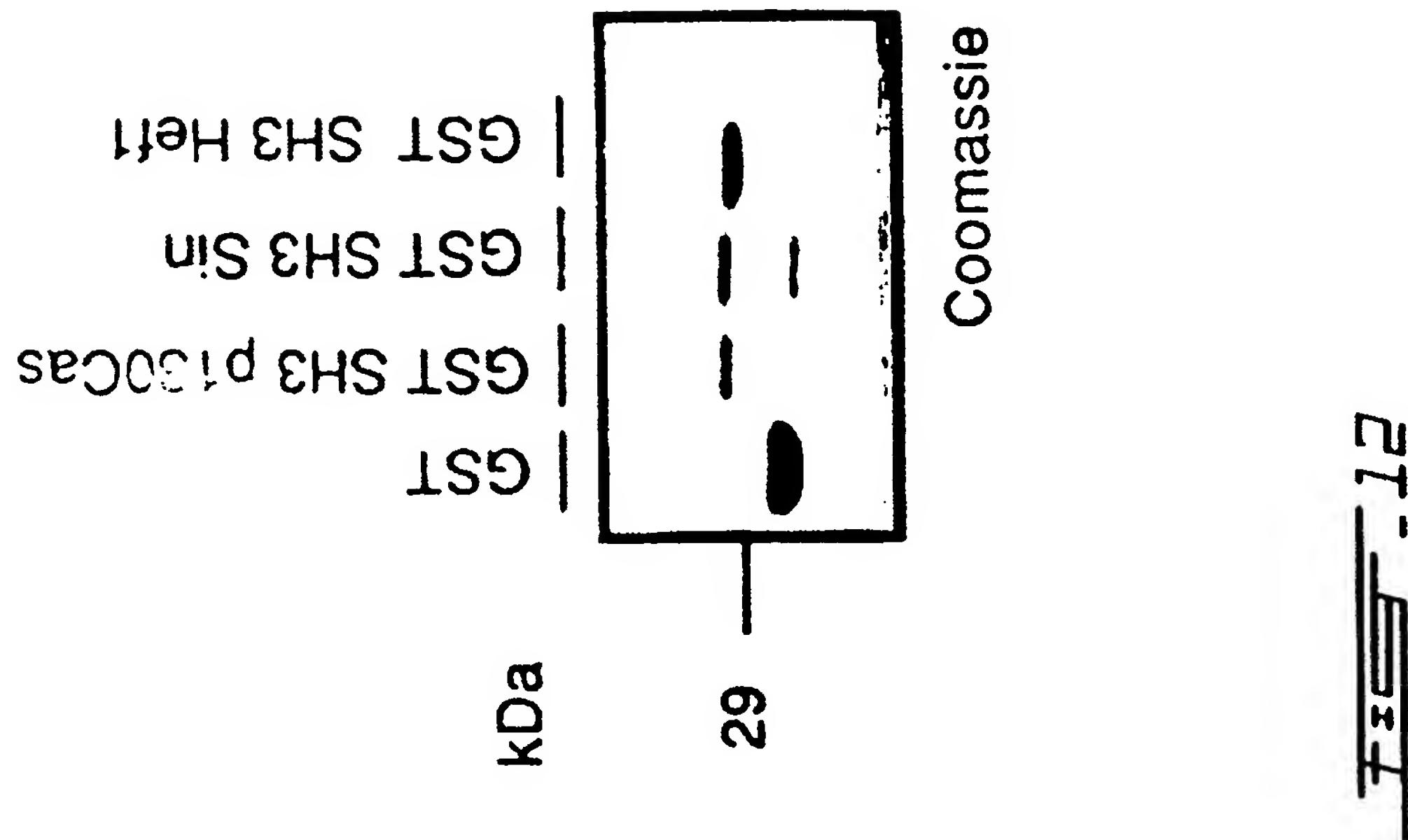
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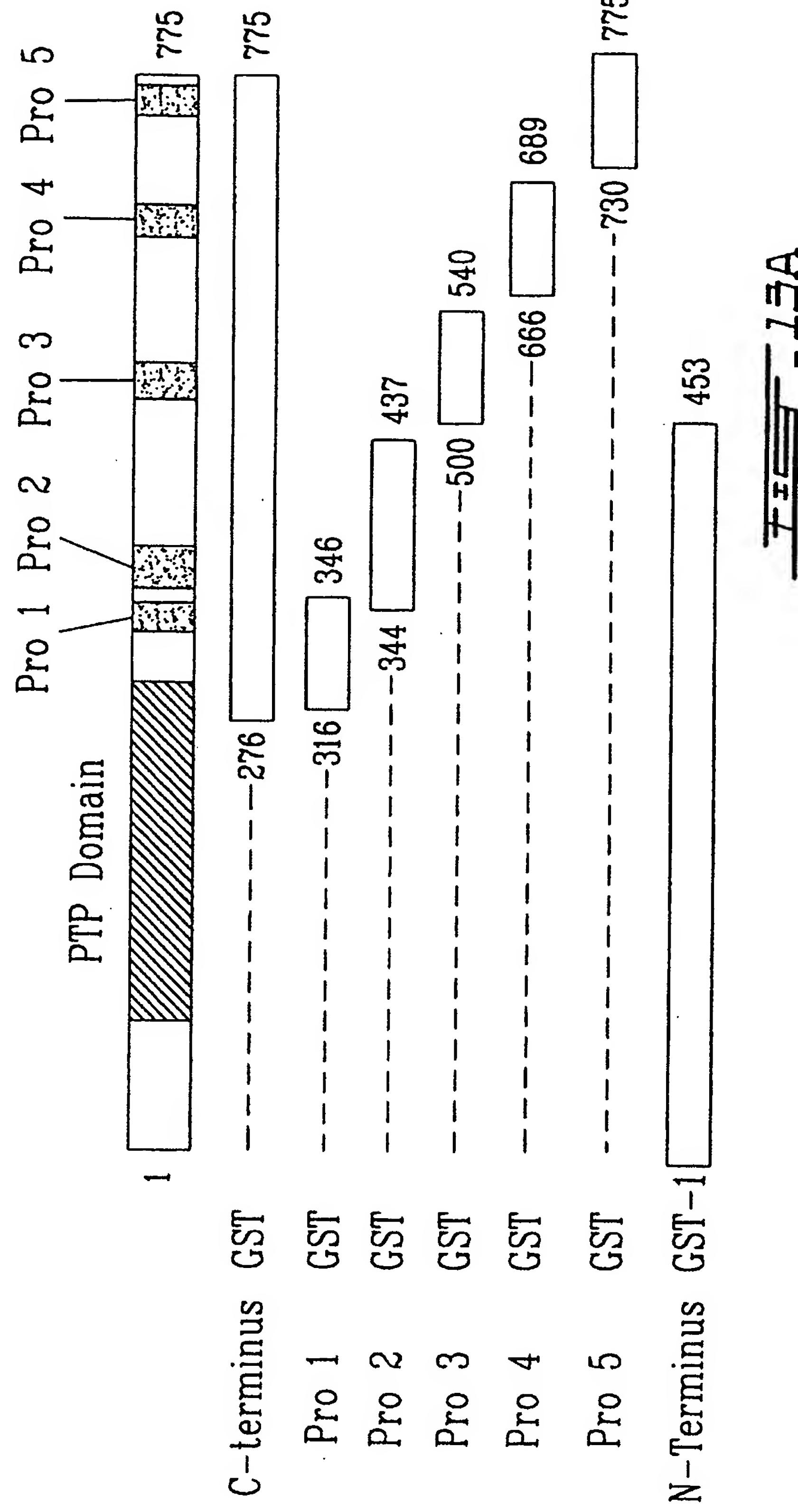


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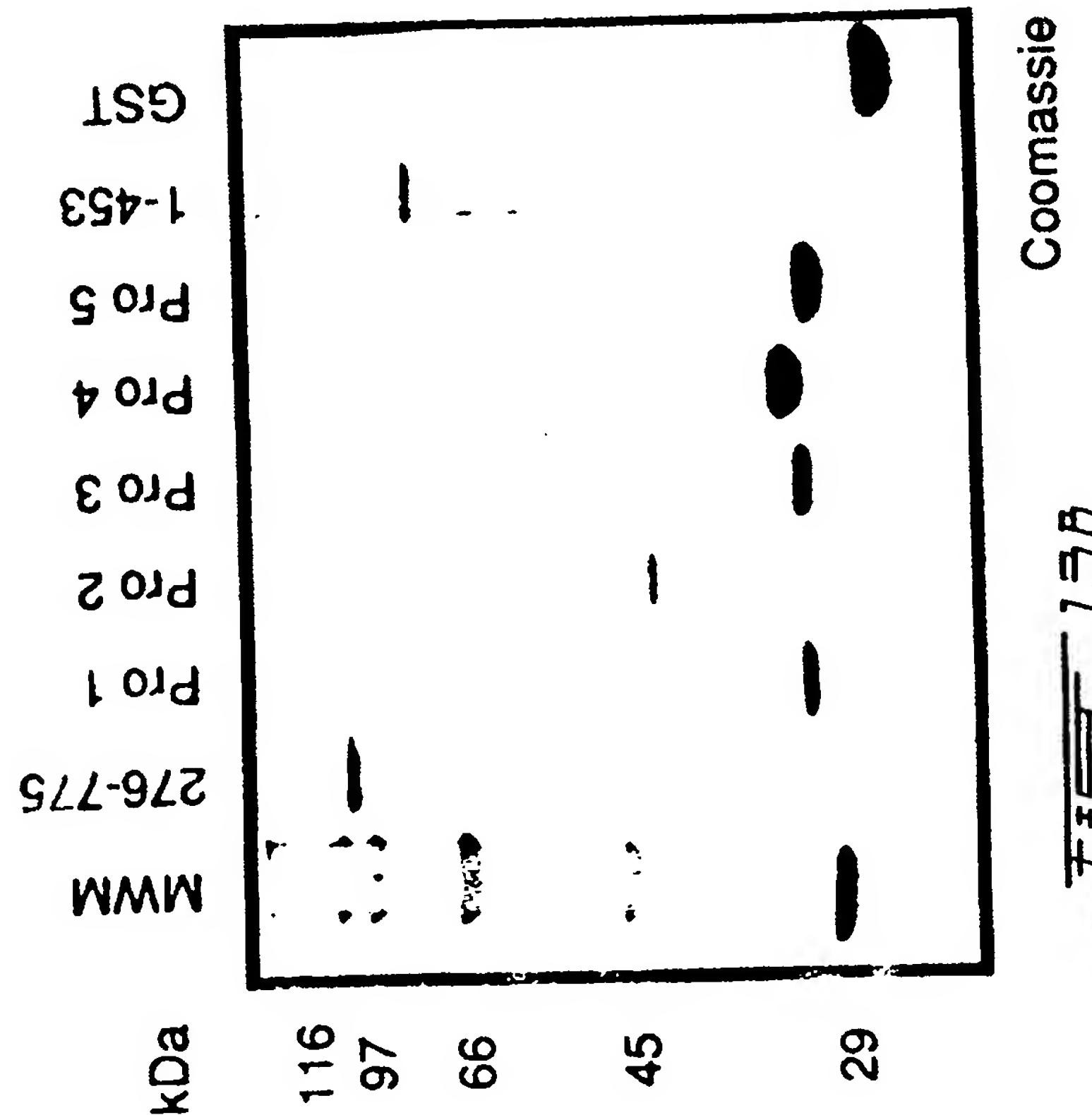
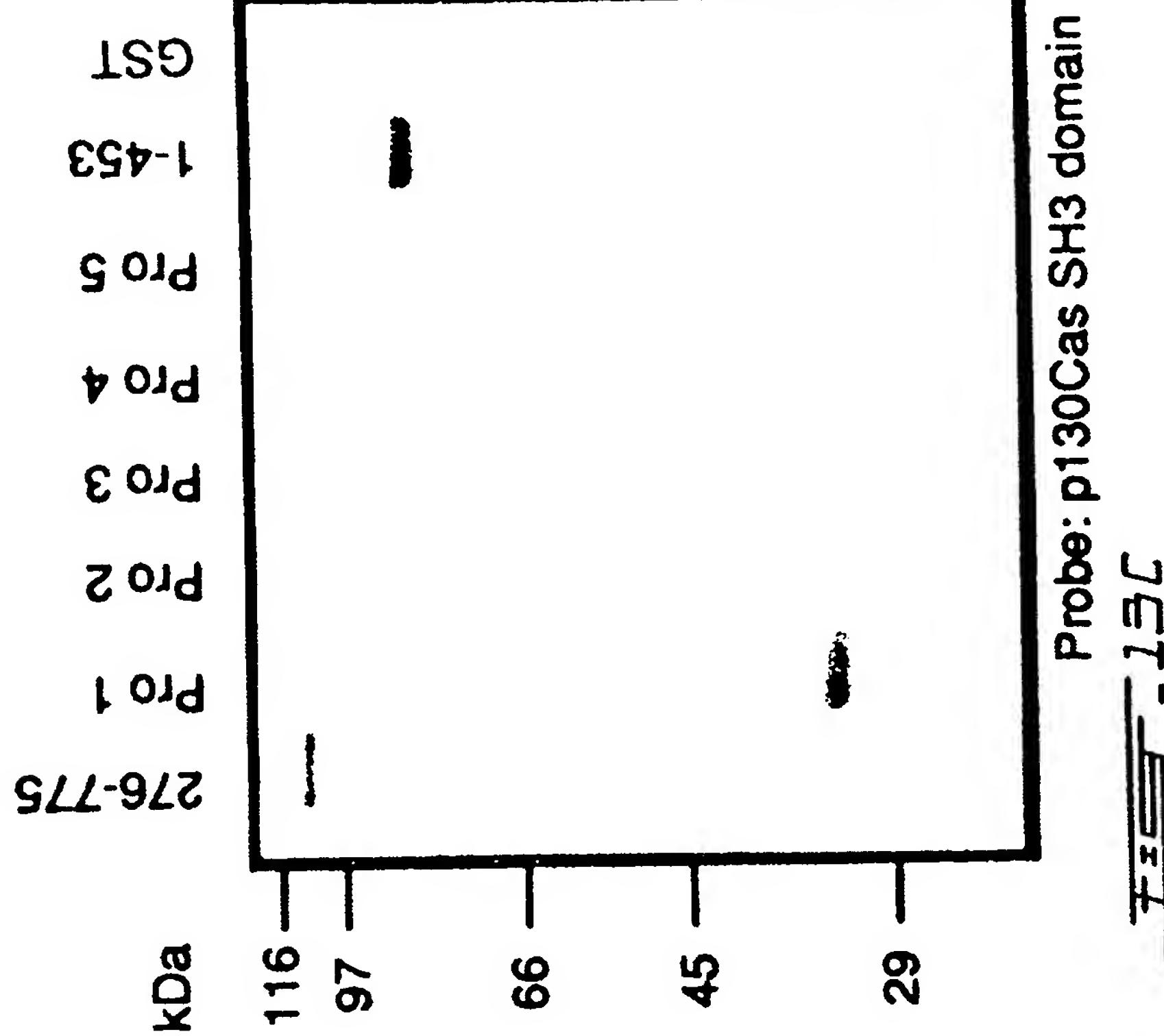
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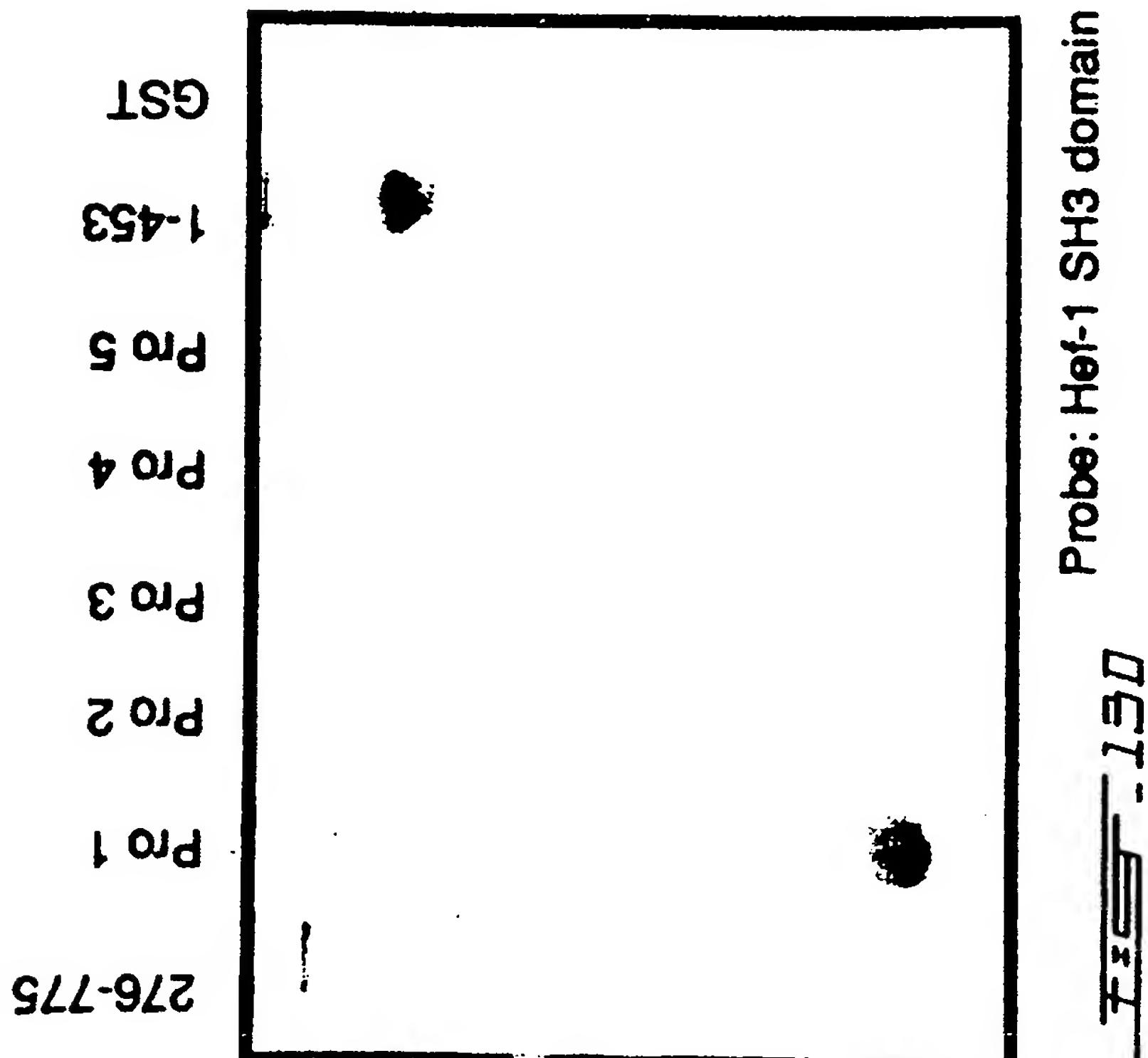
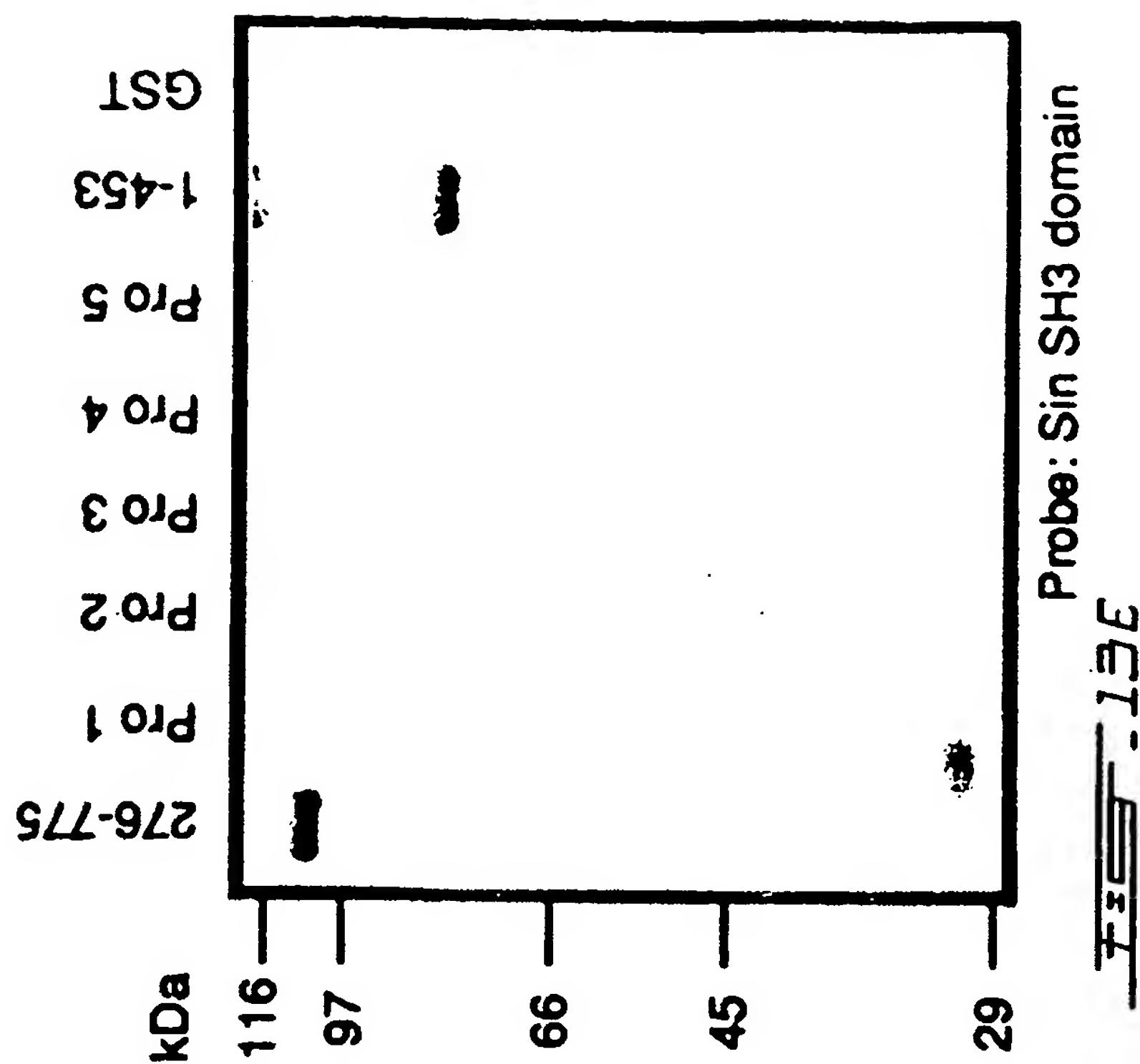
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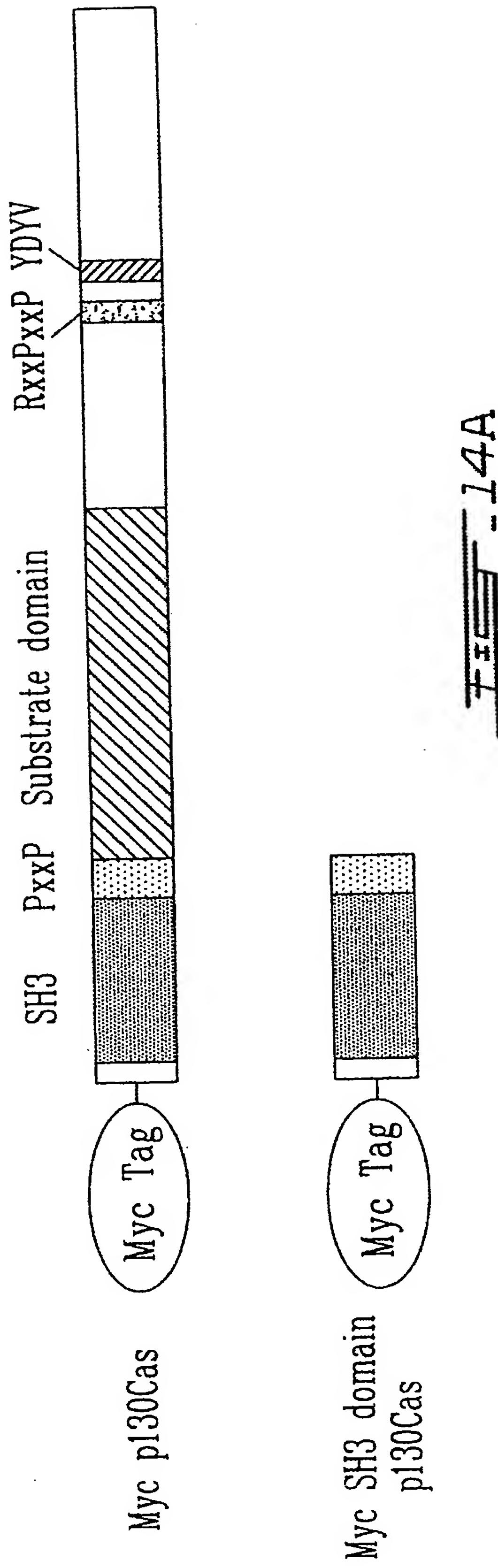
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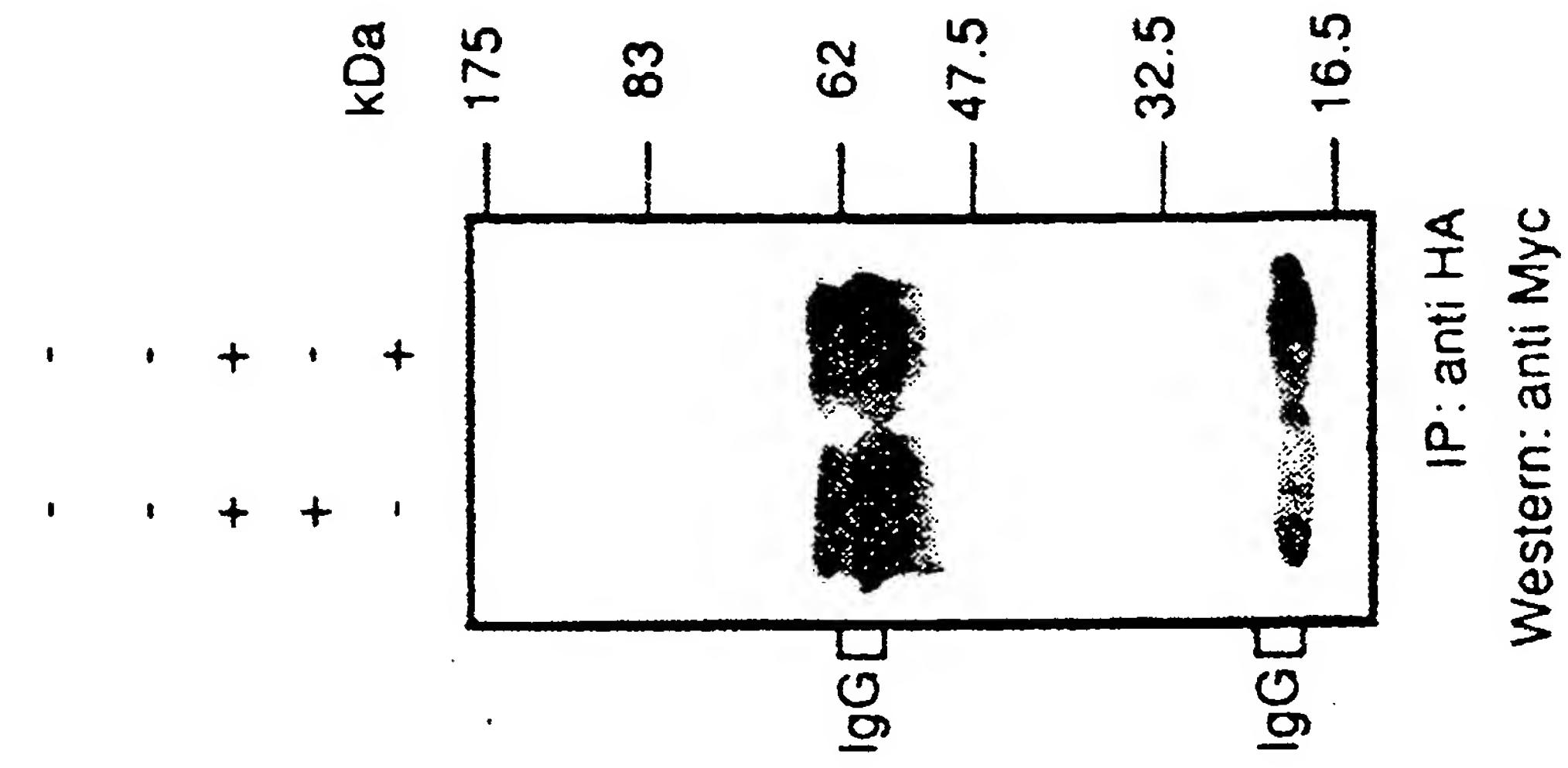


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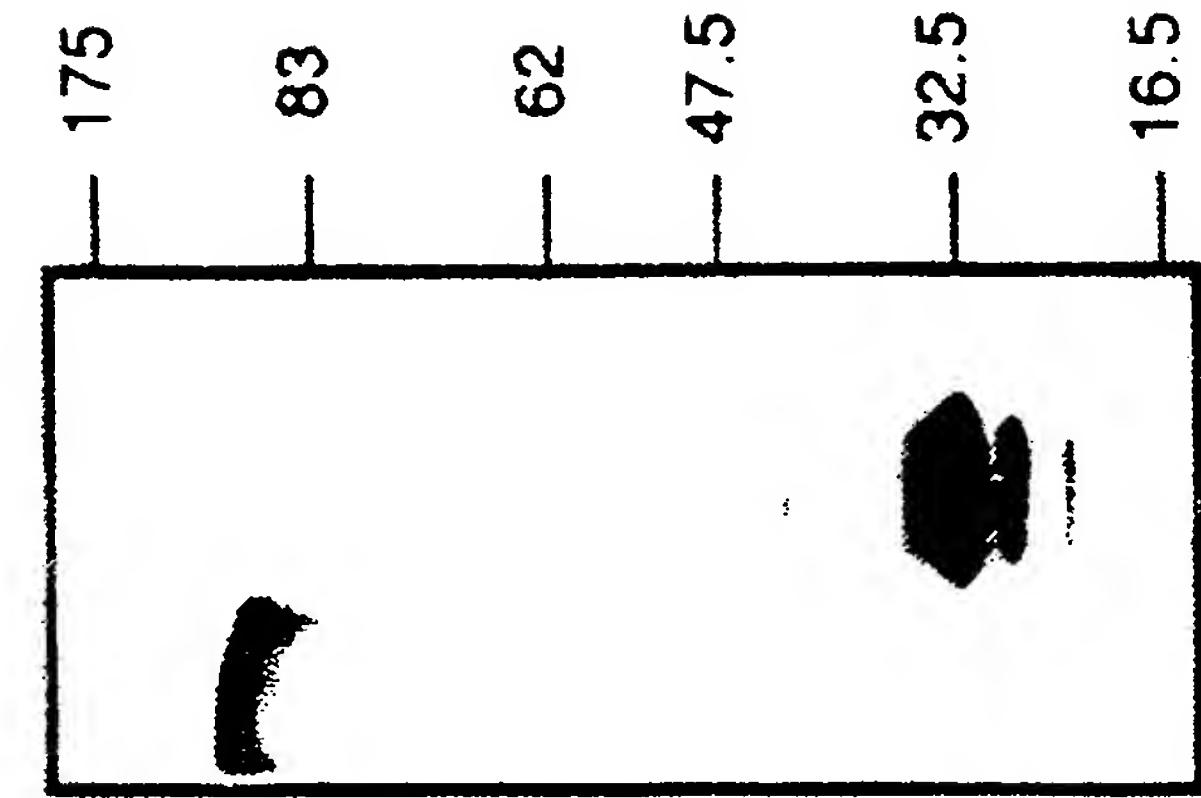
IP: anti HA
Western: anti Myc

IP: anti HA
Western: anti Myc

FIGURE - 14B

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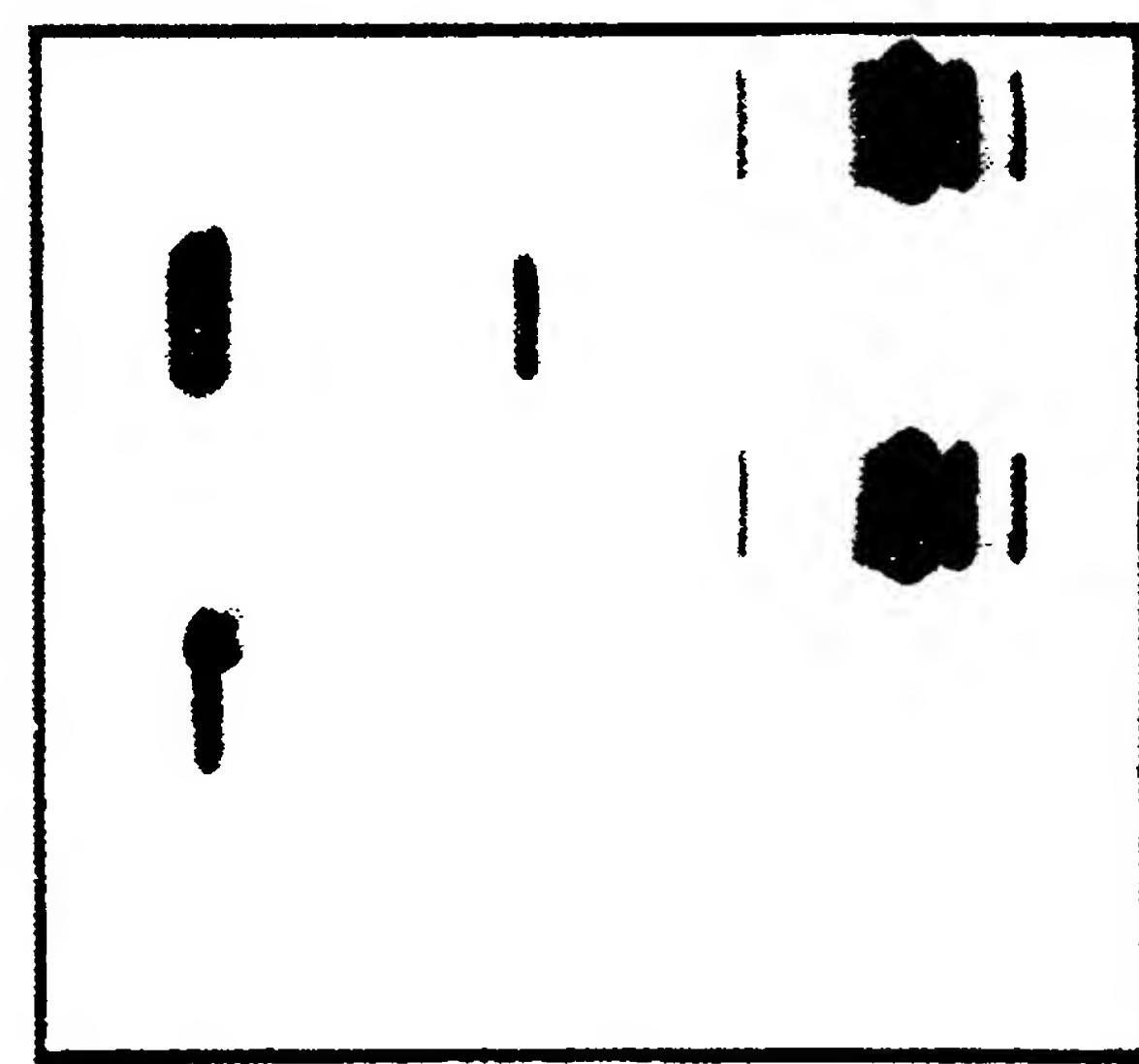
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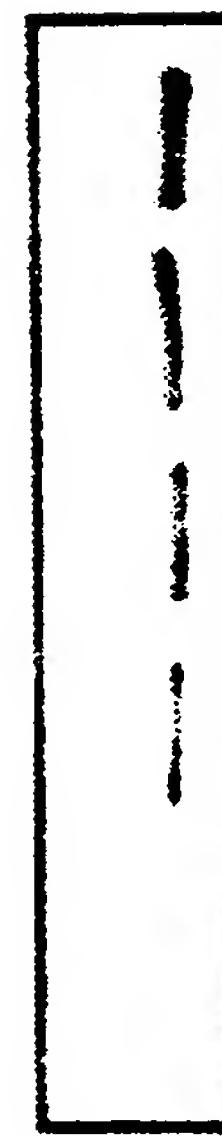
Western: anti Myc



Western: anti HA



Western: anti Myc



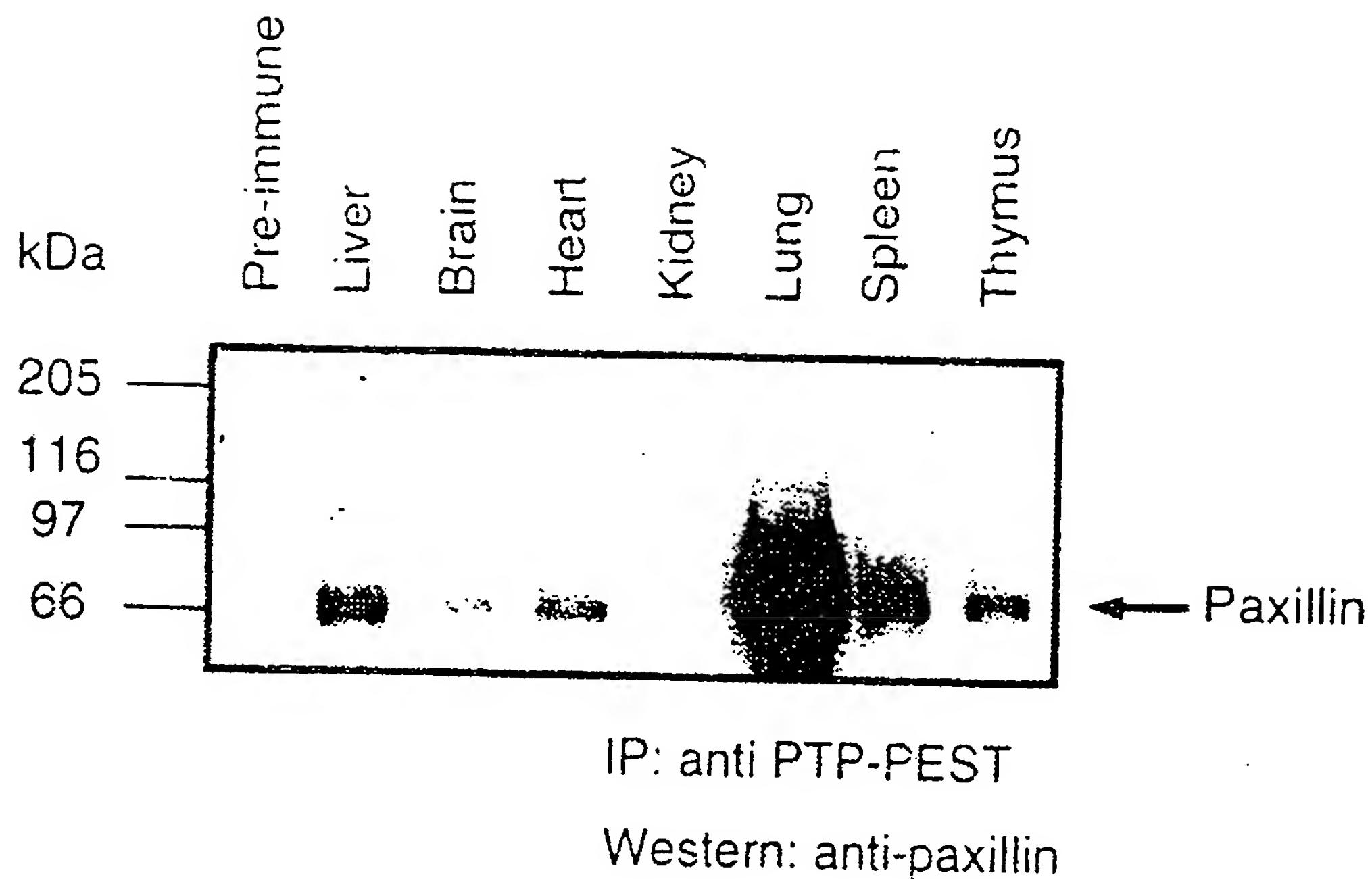
Western: anti HA

Total cell lysates

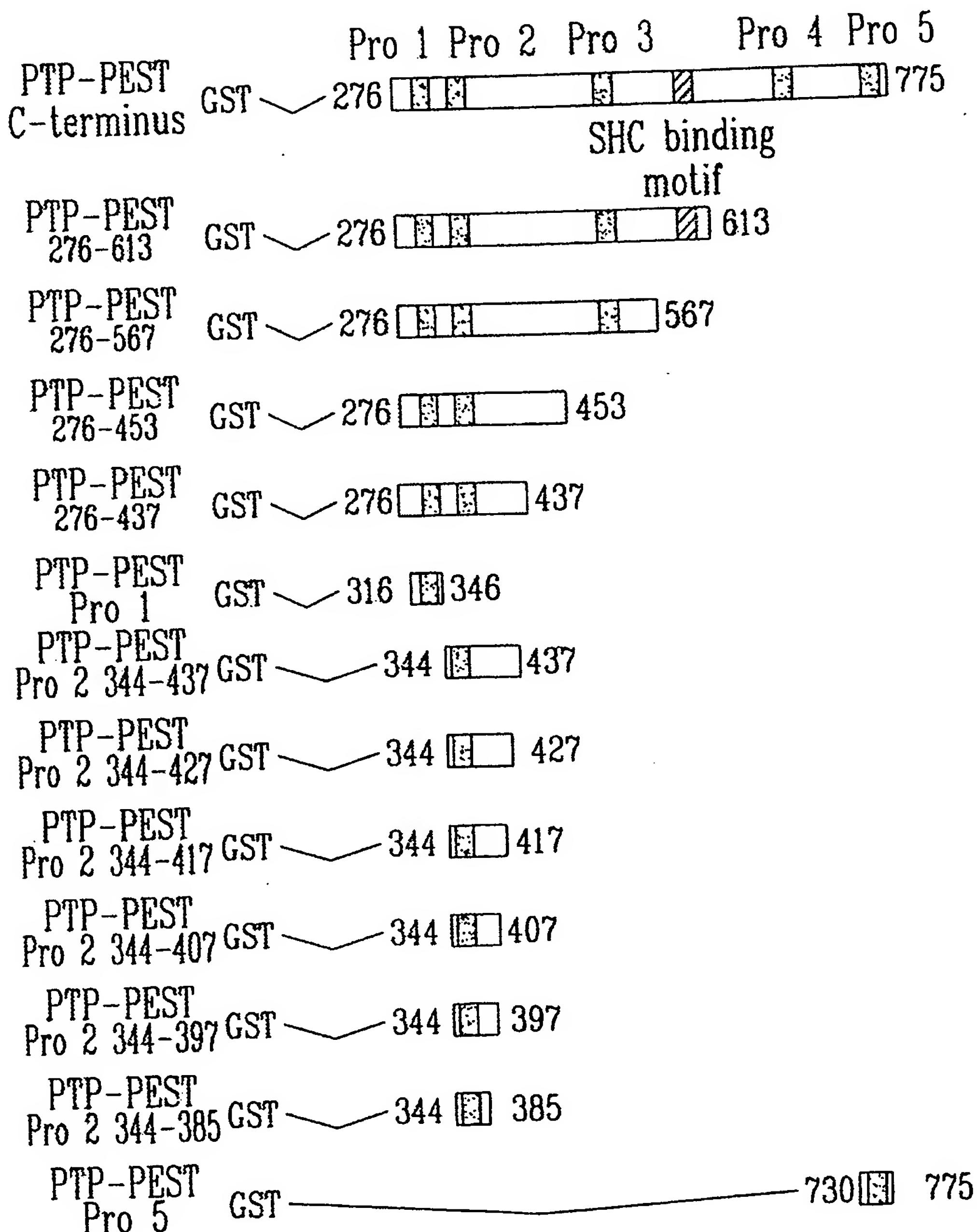
Total cell lysates

756 - 14C

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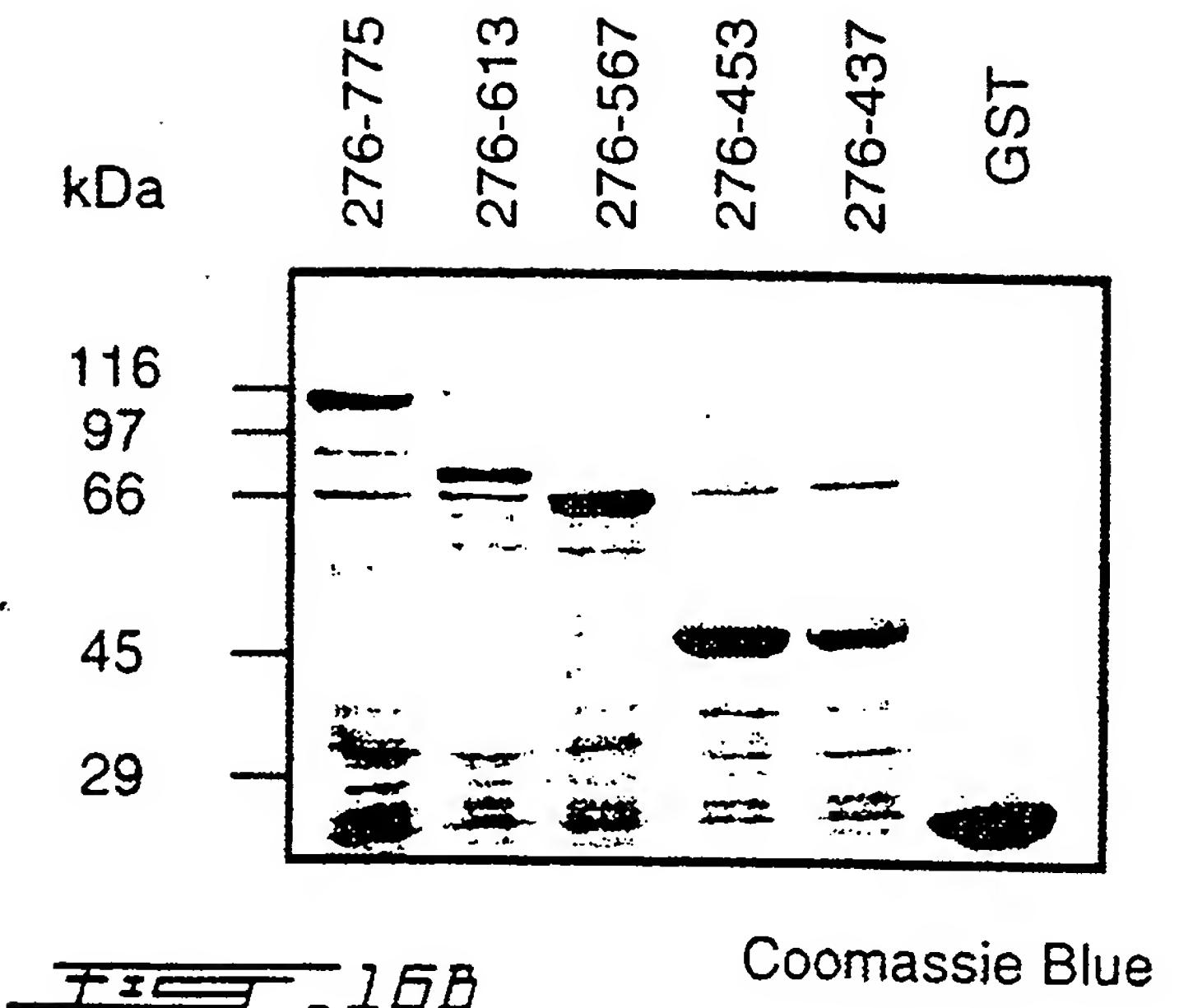
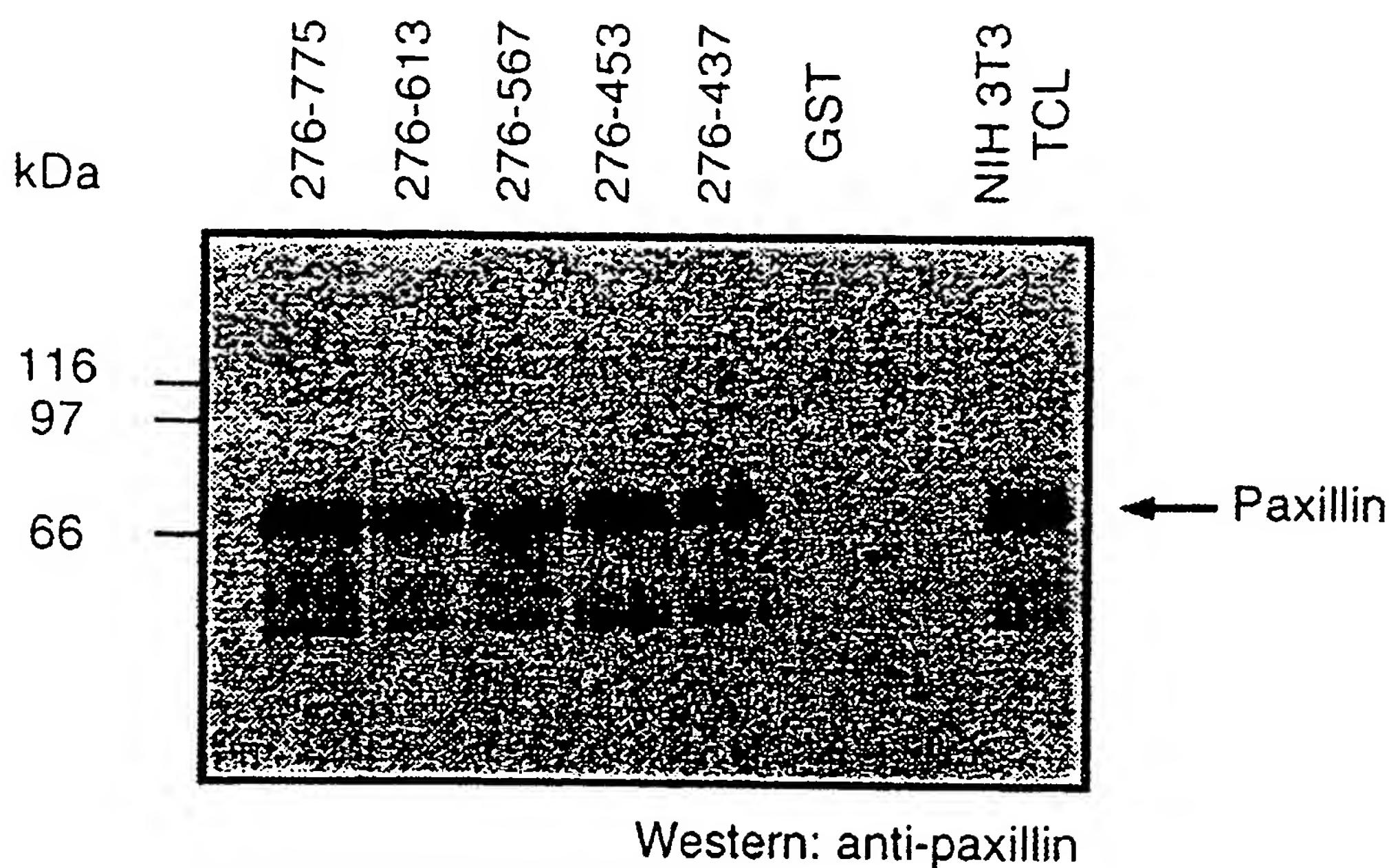
22/39

FIGURE - 16A

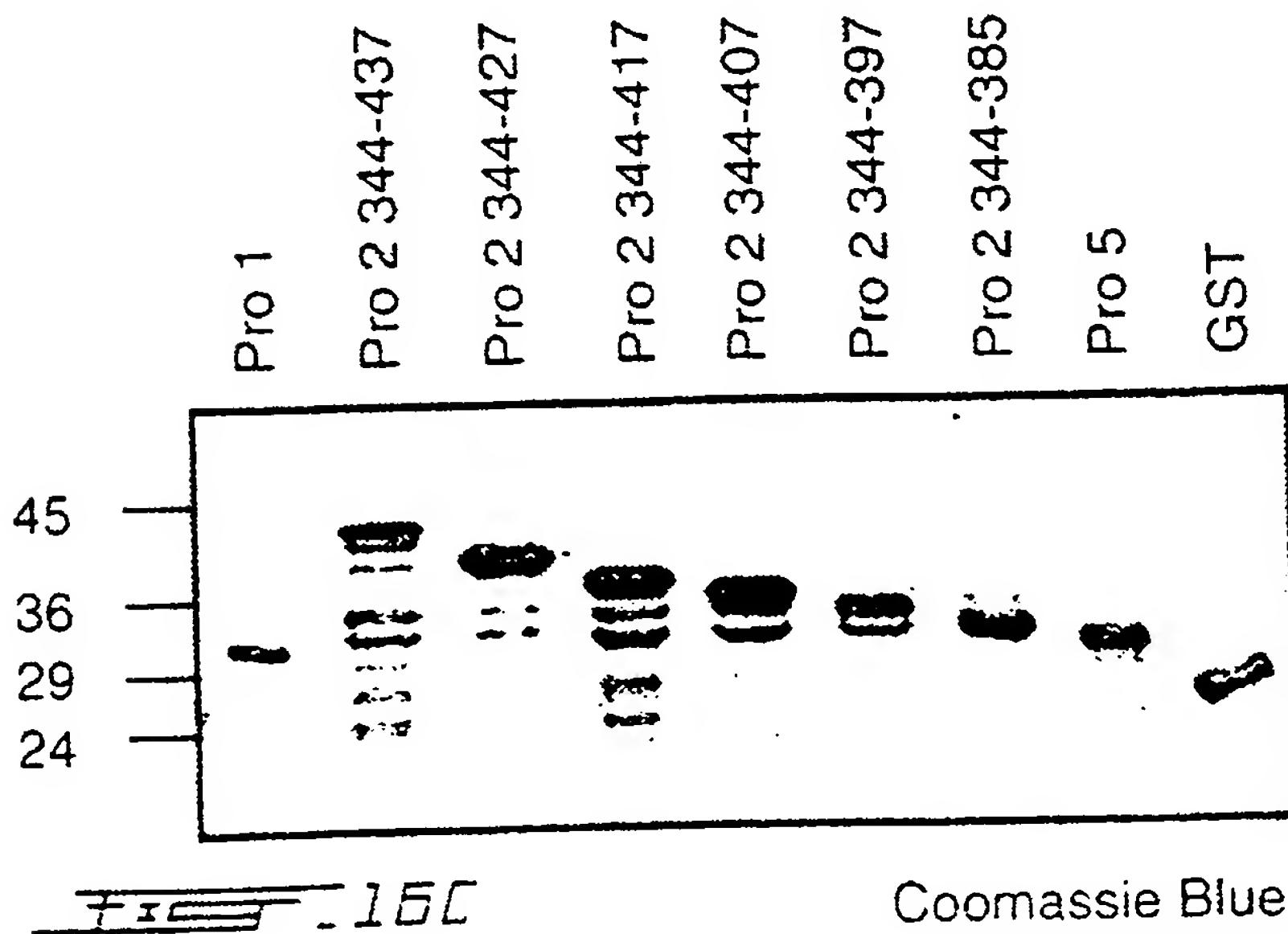
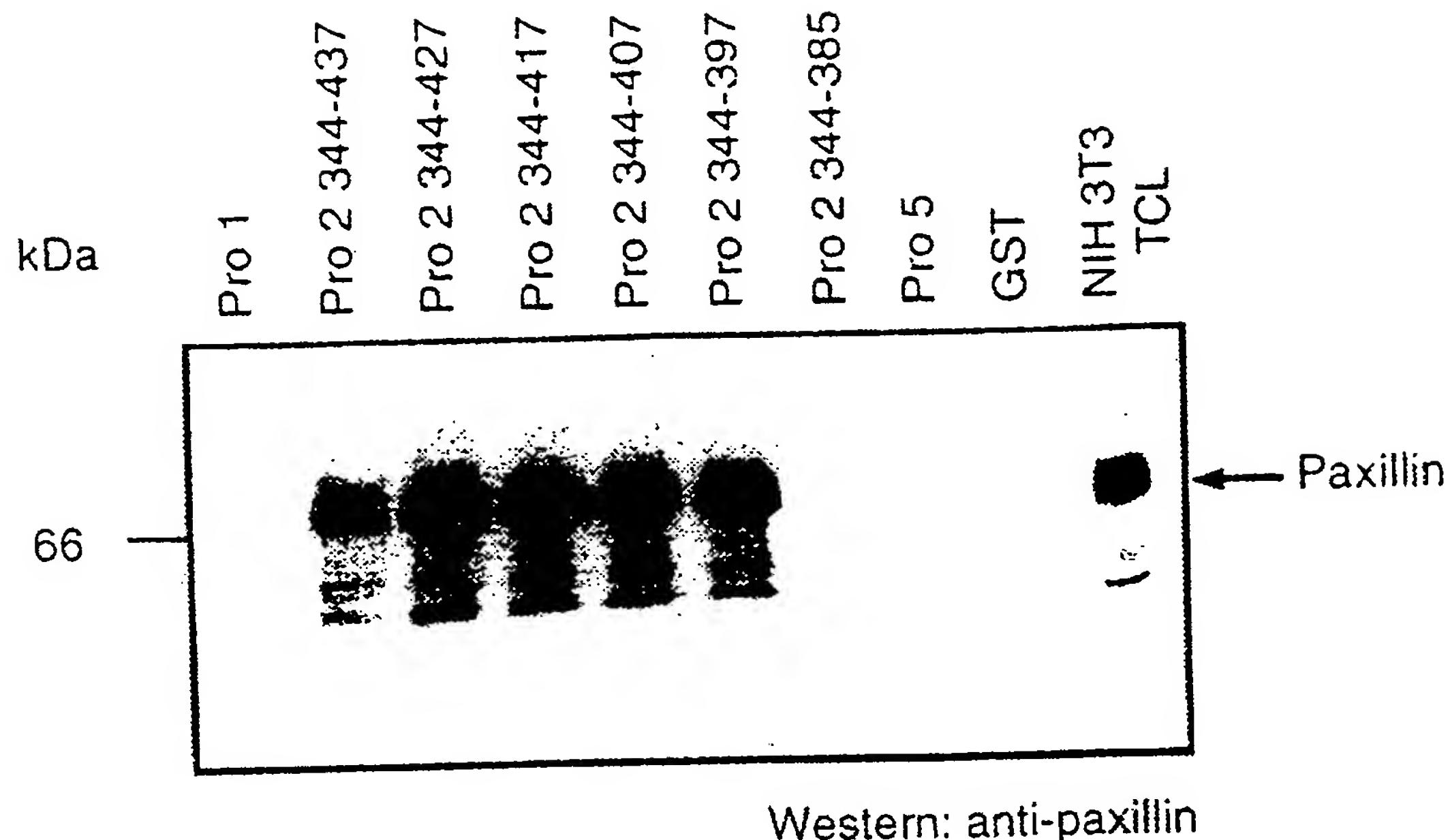
WO 99/61467

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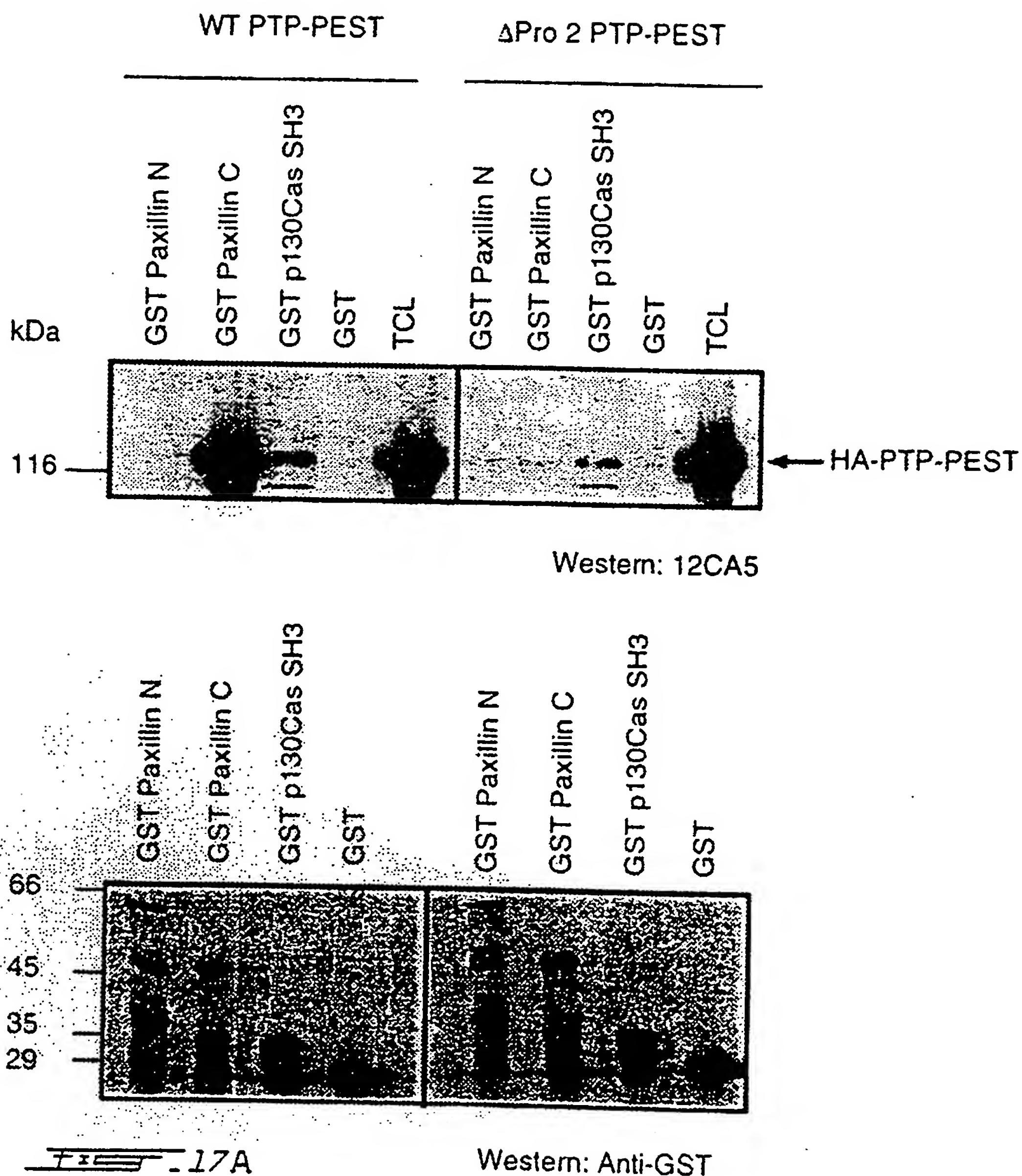
23/39



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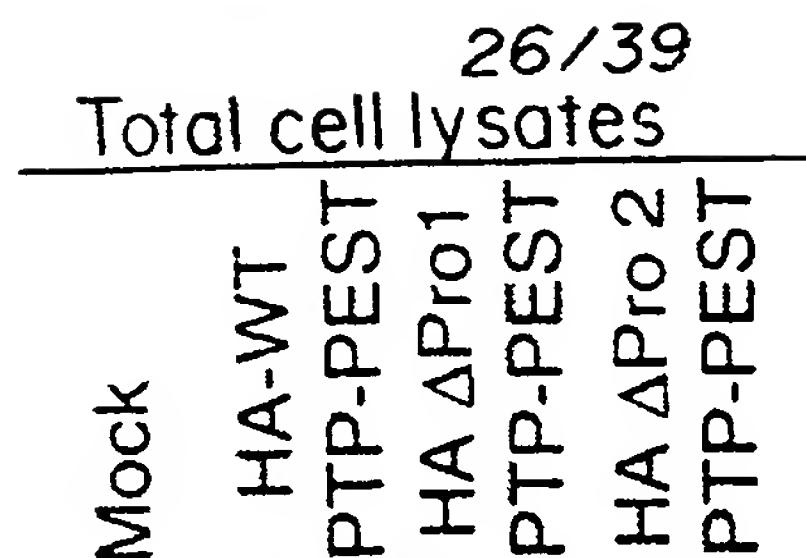
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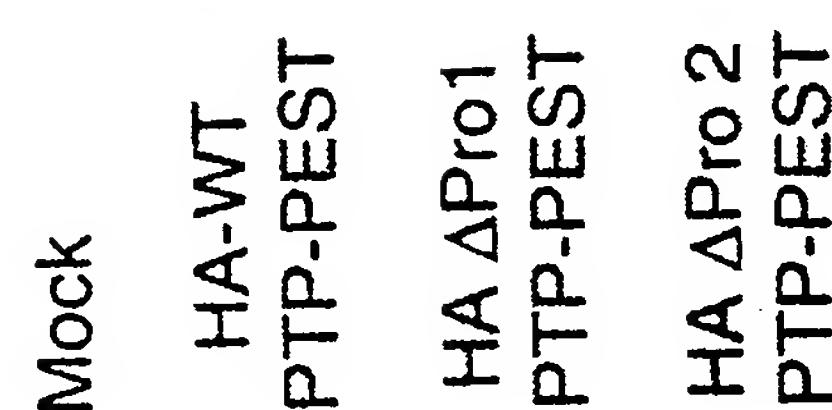
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WO 99/61467

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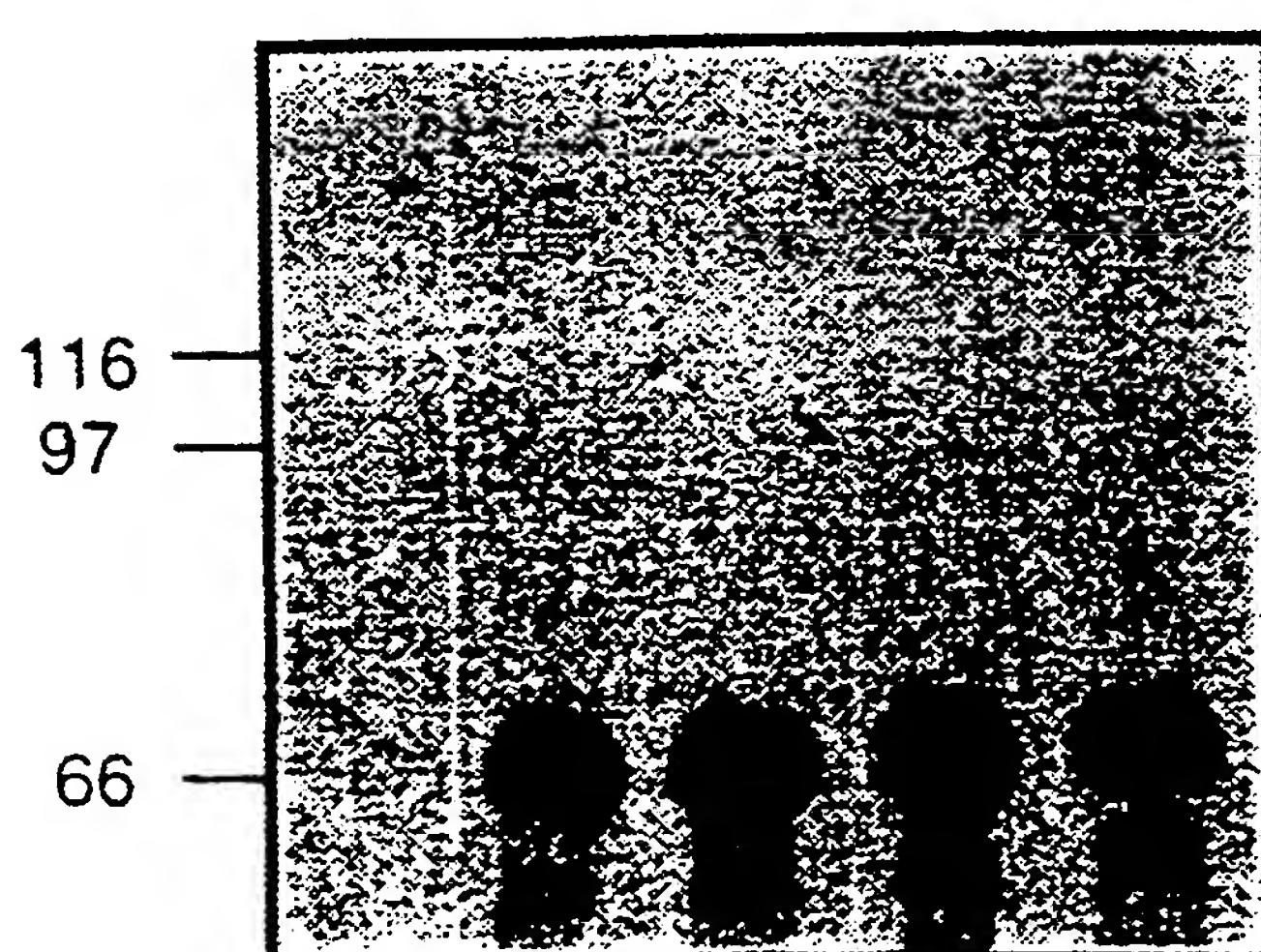


Western: 12CA5



← HA-PTP-PEST

IP: anti-Paxillin
Western: 12CA5



← Paxillin
IP: anti-Paxillin
Western: anti-Paxillin

FIG - 17B

W-T 355-P E P H P V P P I L T P S P P S A F P-374

P358A 355-P E P E H P V P P I L T P S P P S A F P-374

P360A 355-P E P H V P P I L T P S P P S A F P-374

P362A 355-P E P H P V P I L T P S P P S A F P-374

P363A 355-P E P H P V P I L T P S P P S A F P-374

P367A 355-P E P H P V P P I L T S P P S A F P-374 27/39

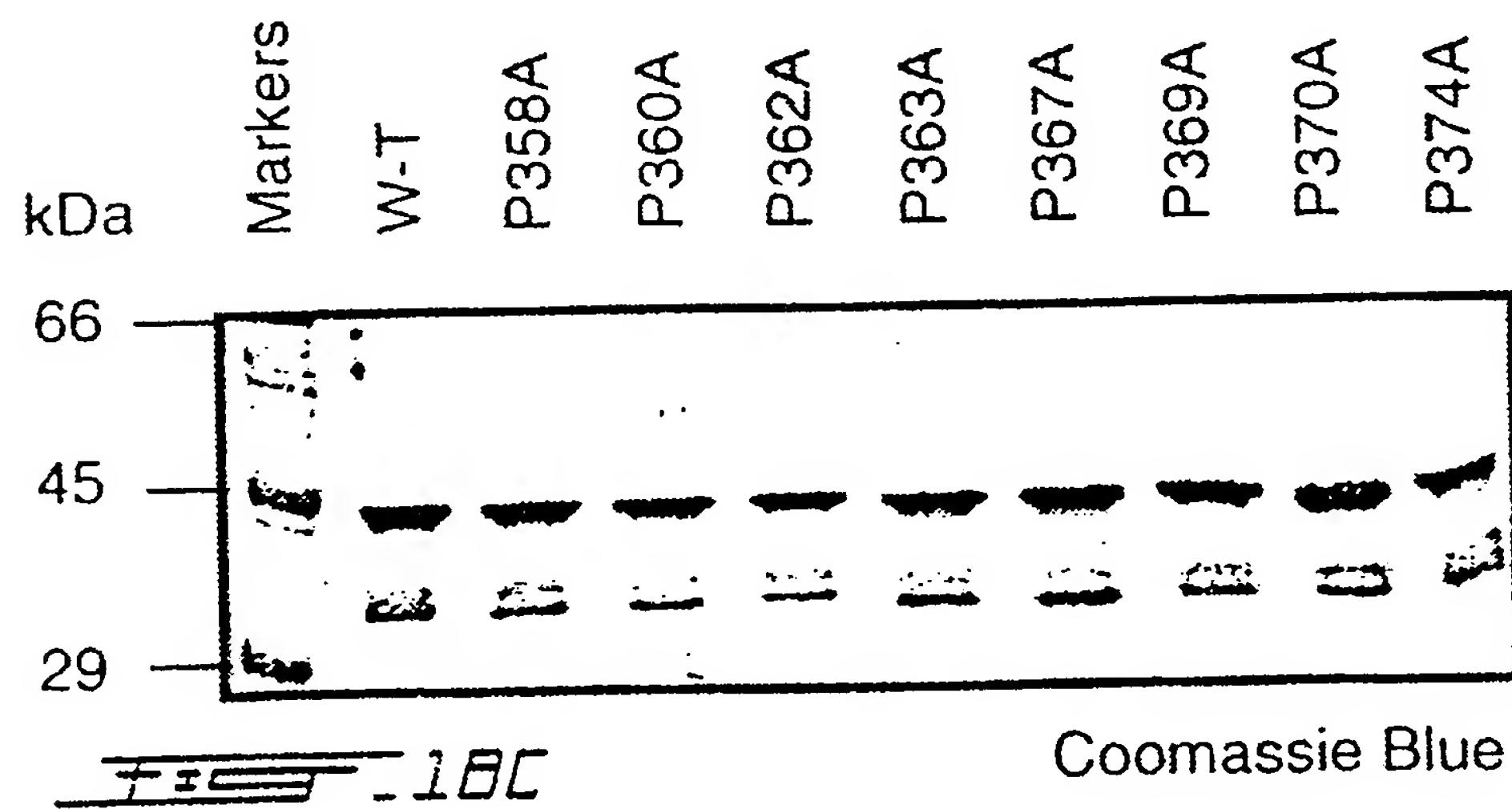
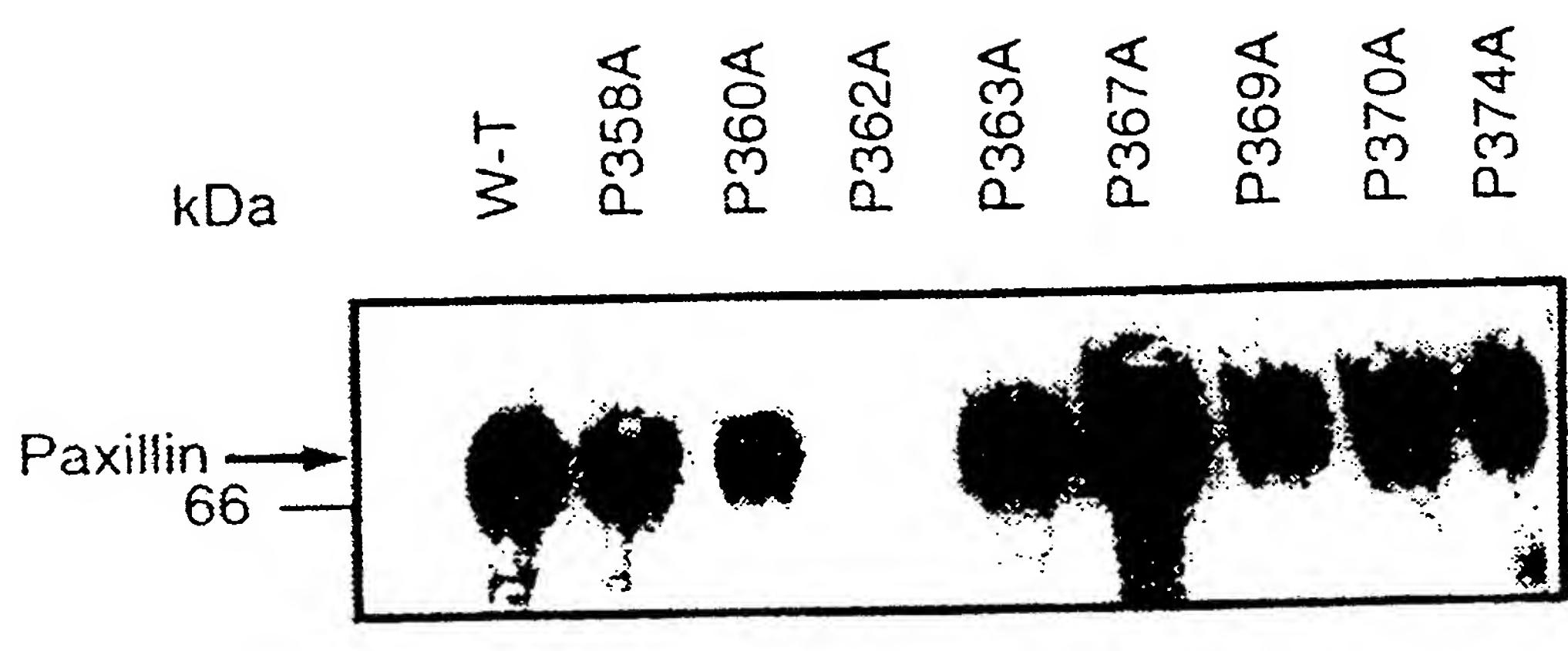
P369A 355-P E P H P V P P I L T P S P S A F P-374

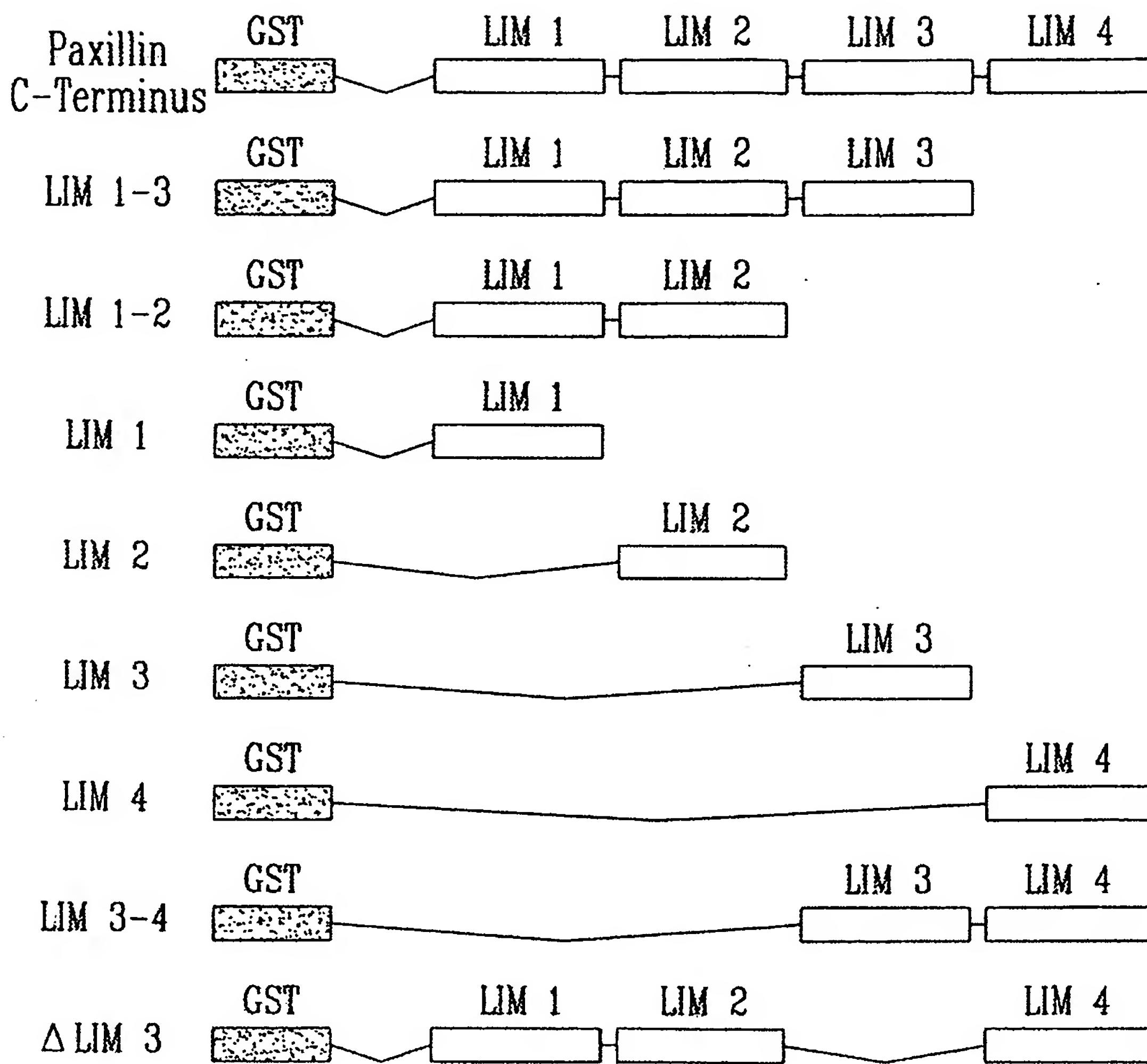
P370A 355-P E P H P V P P I L T P S P S A F P-374

P374A 355-P E P H P V P P I L T P S P P S A F A 374

~~7~~
~~7~~
~~7~~-18A

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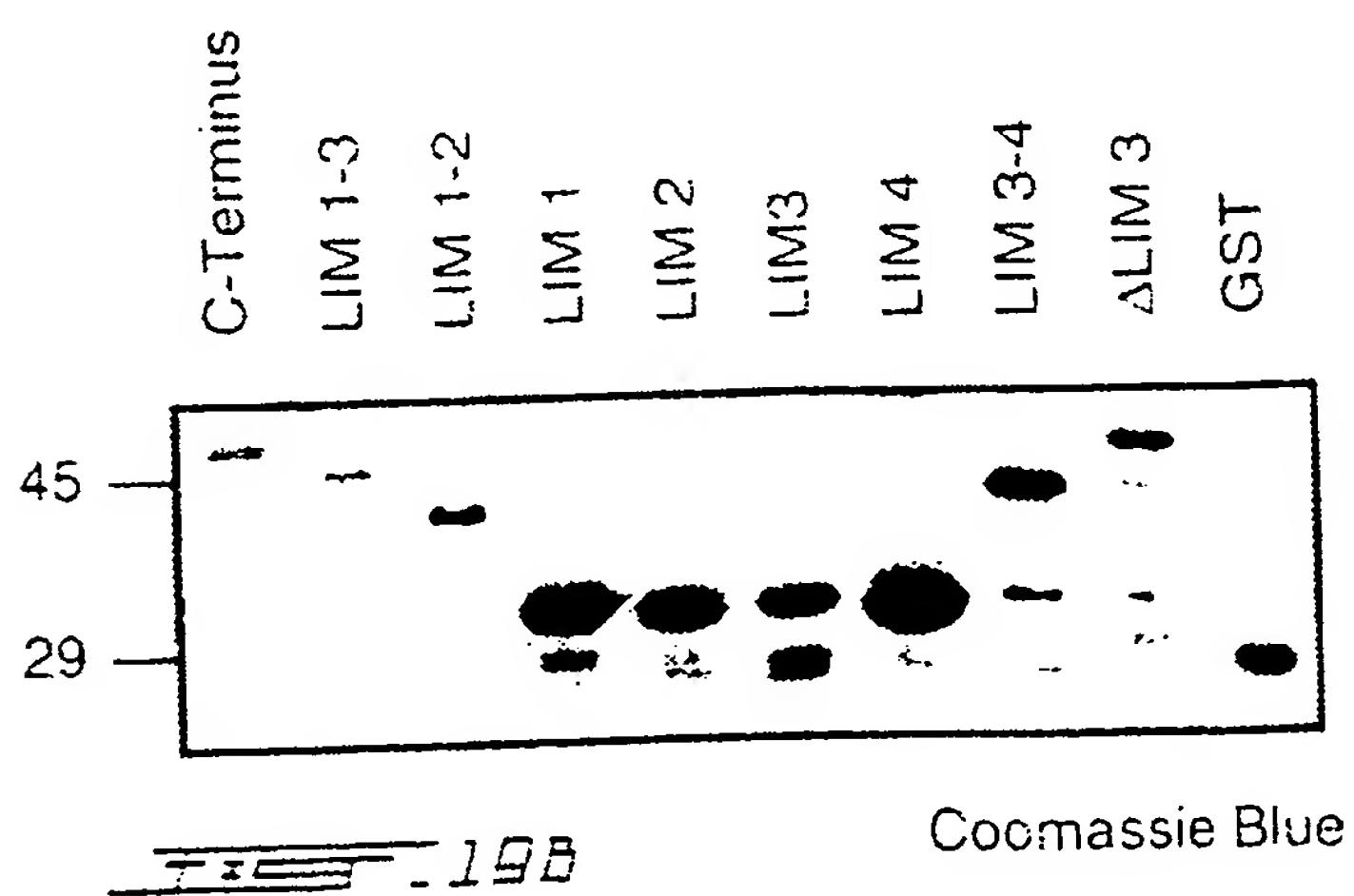
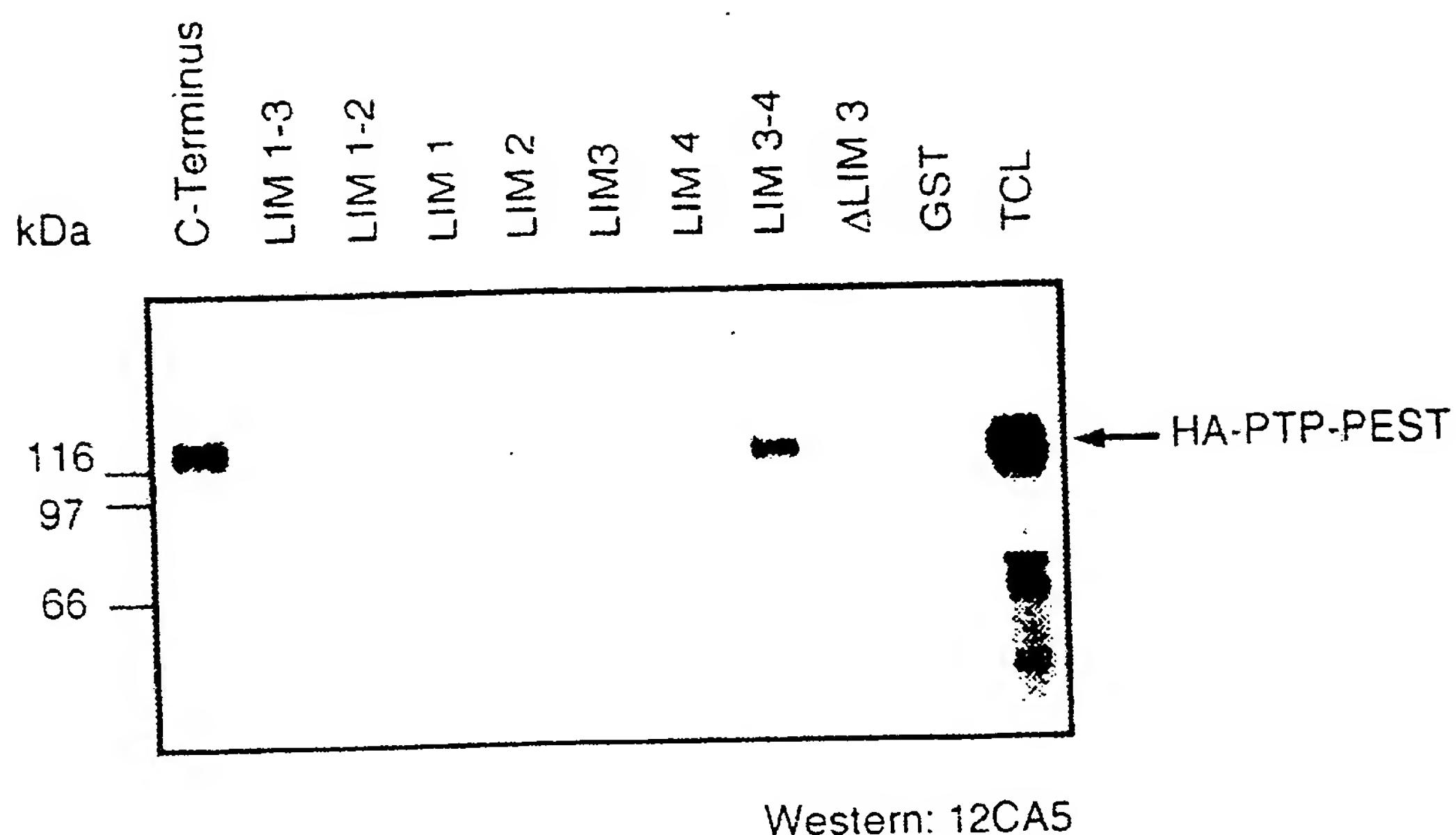


~~FIGURE - 19A~~

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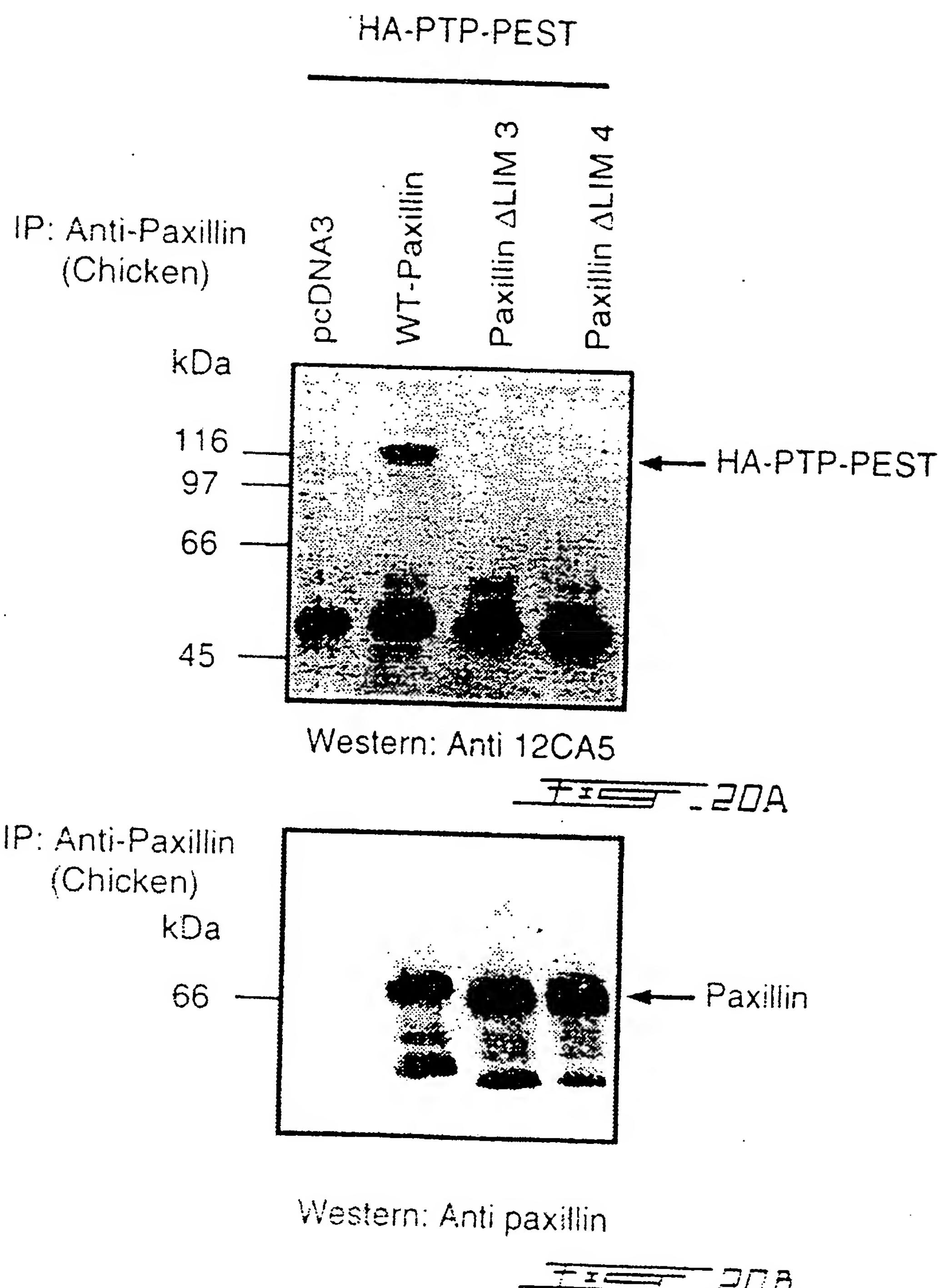
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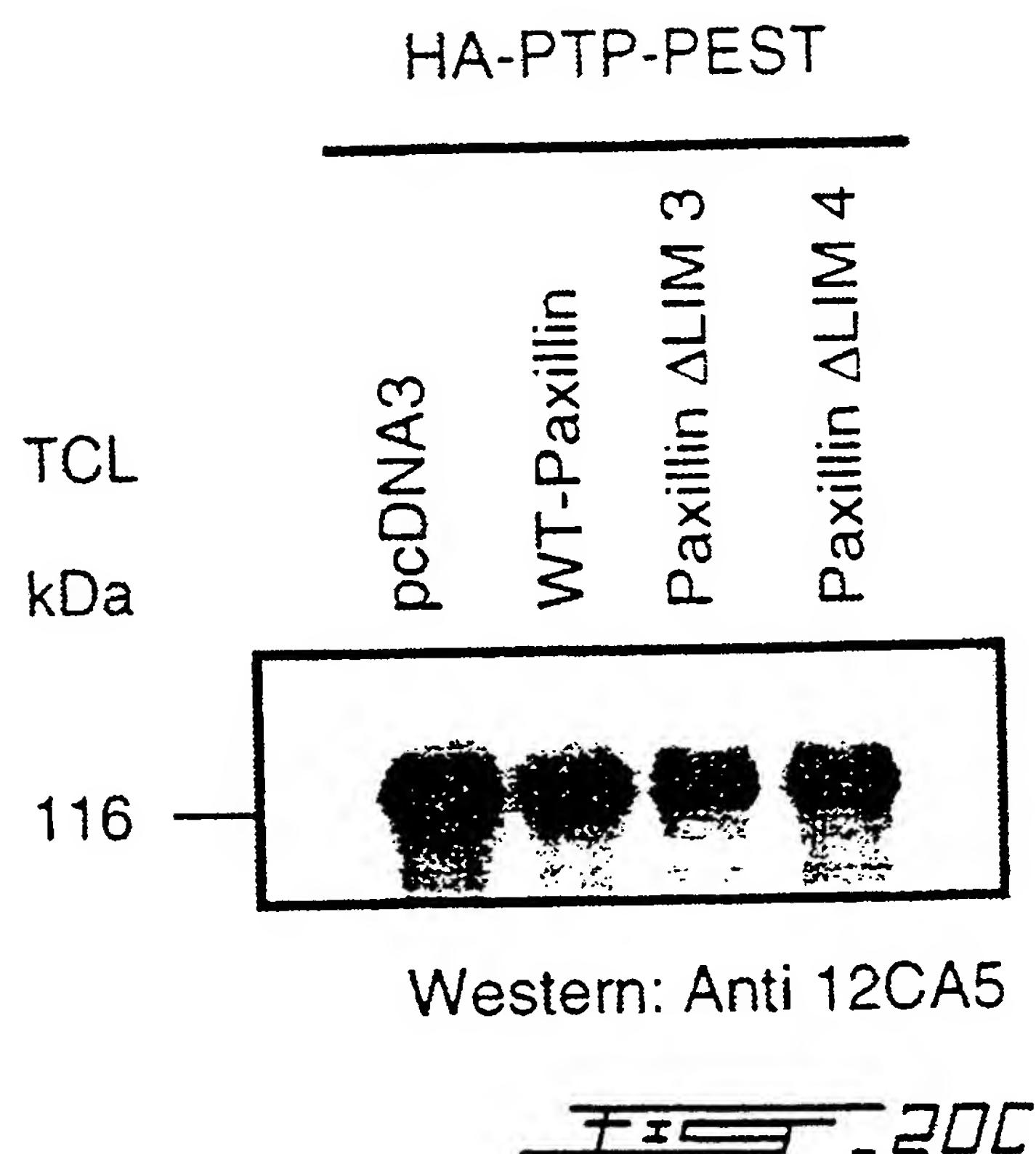


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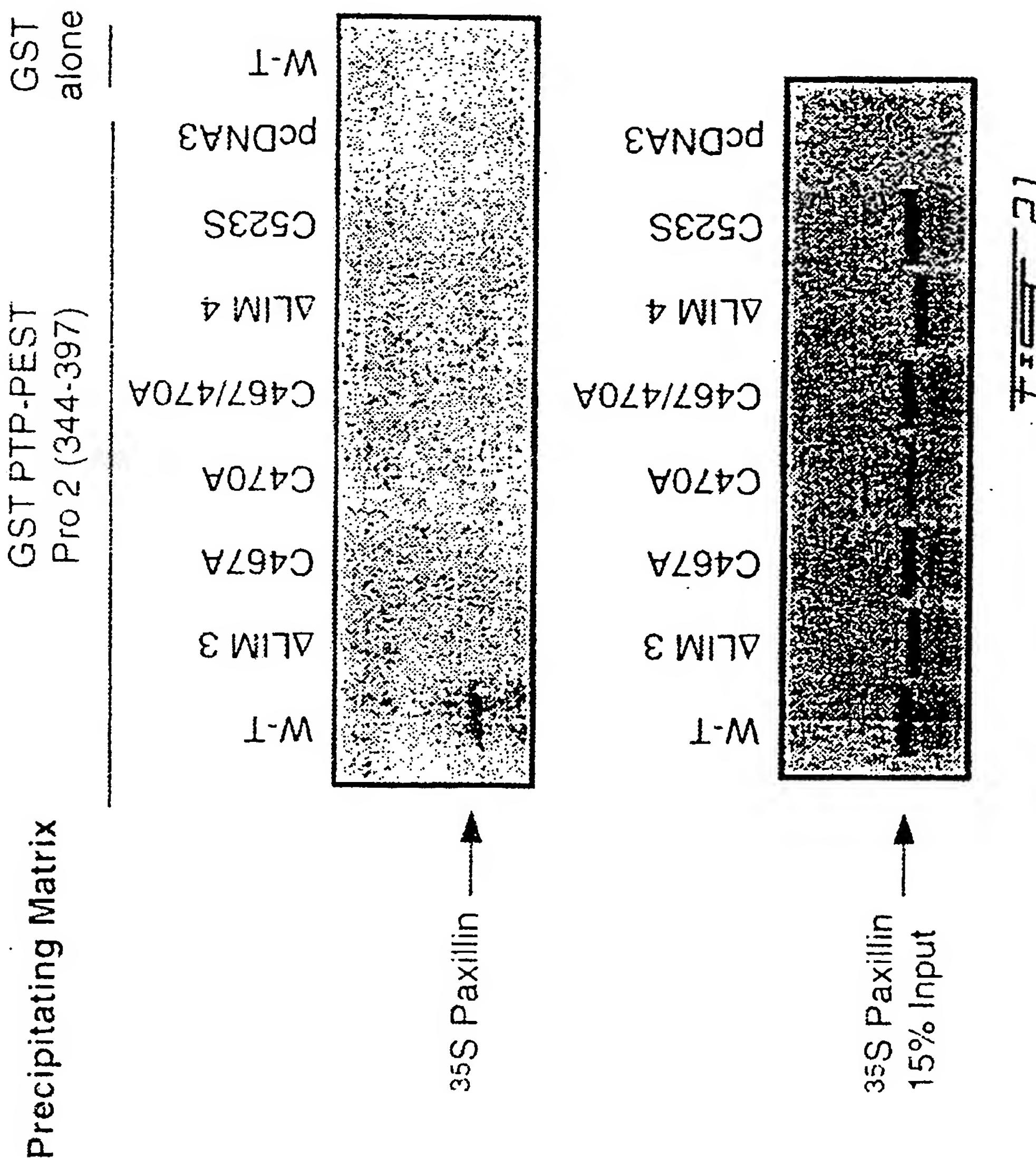
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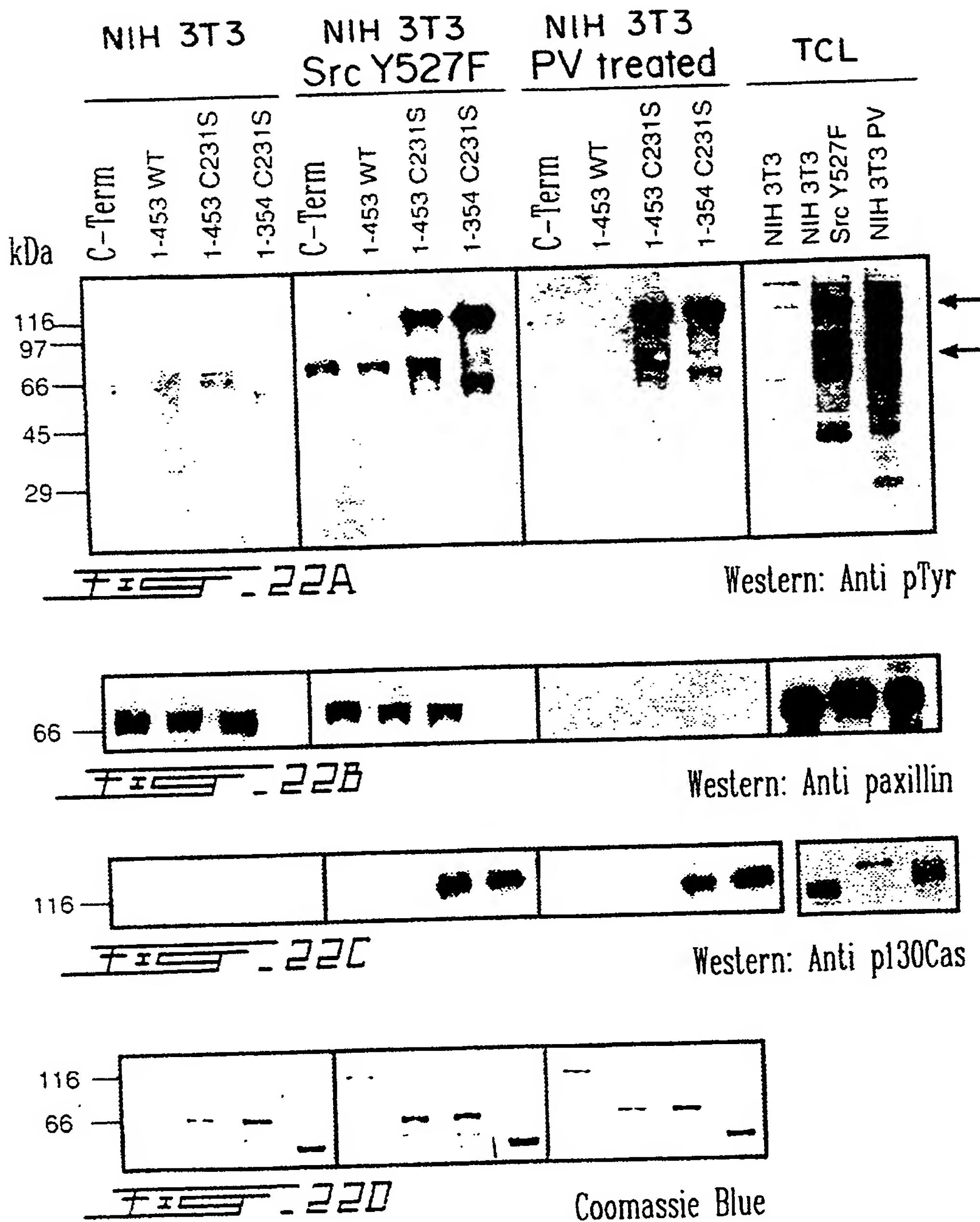
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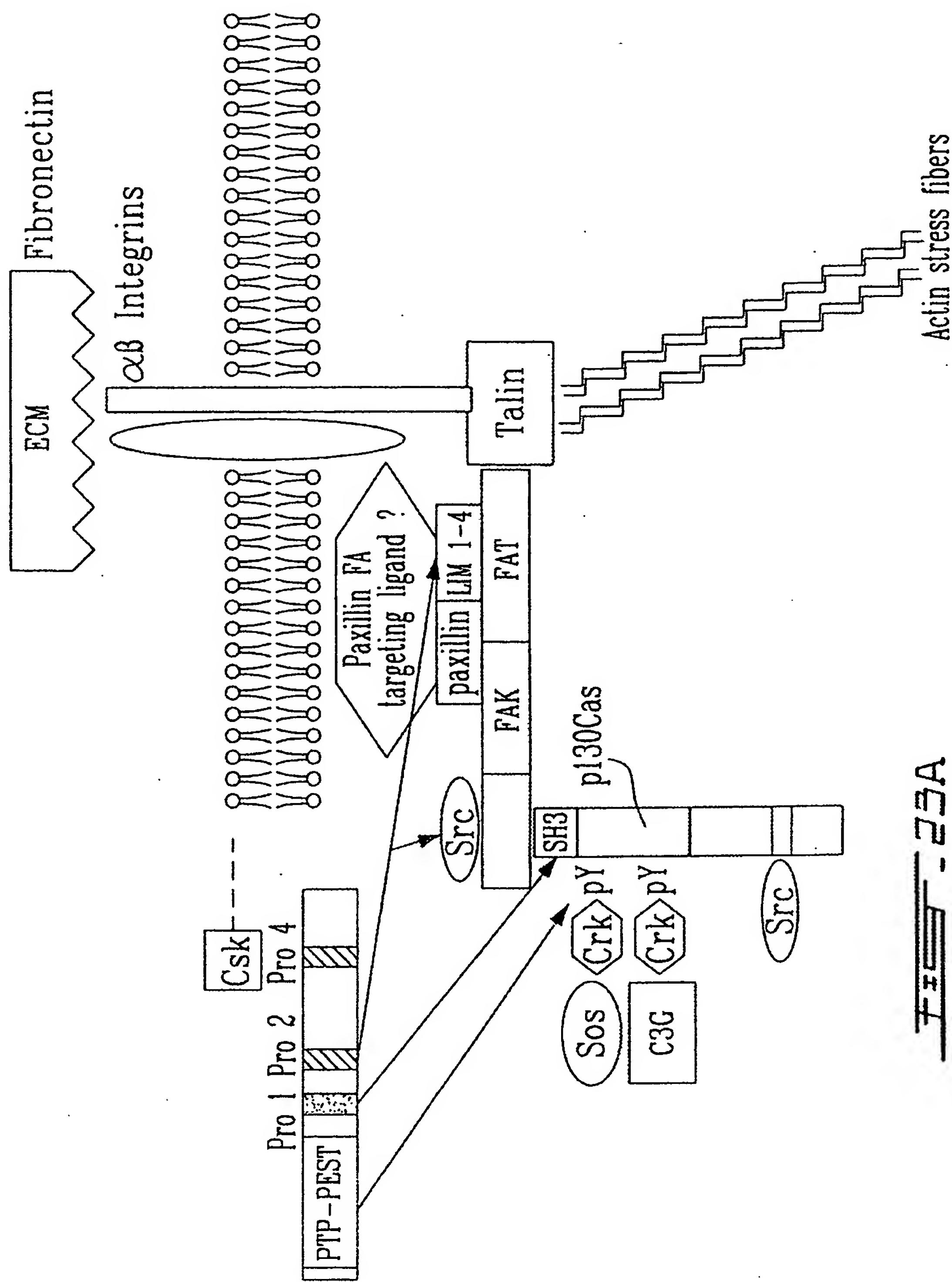
33/39



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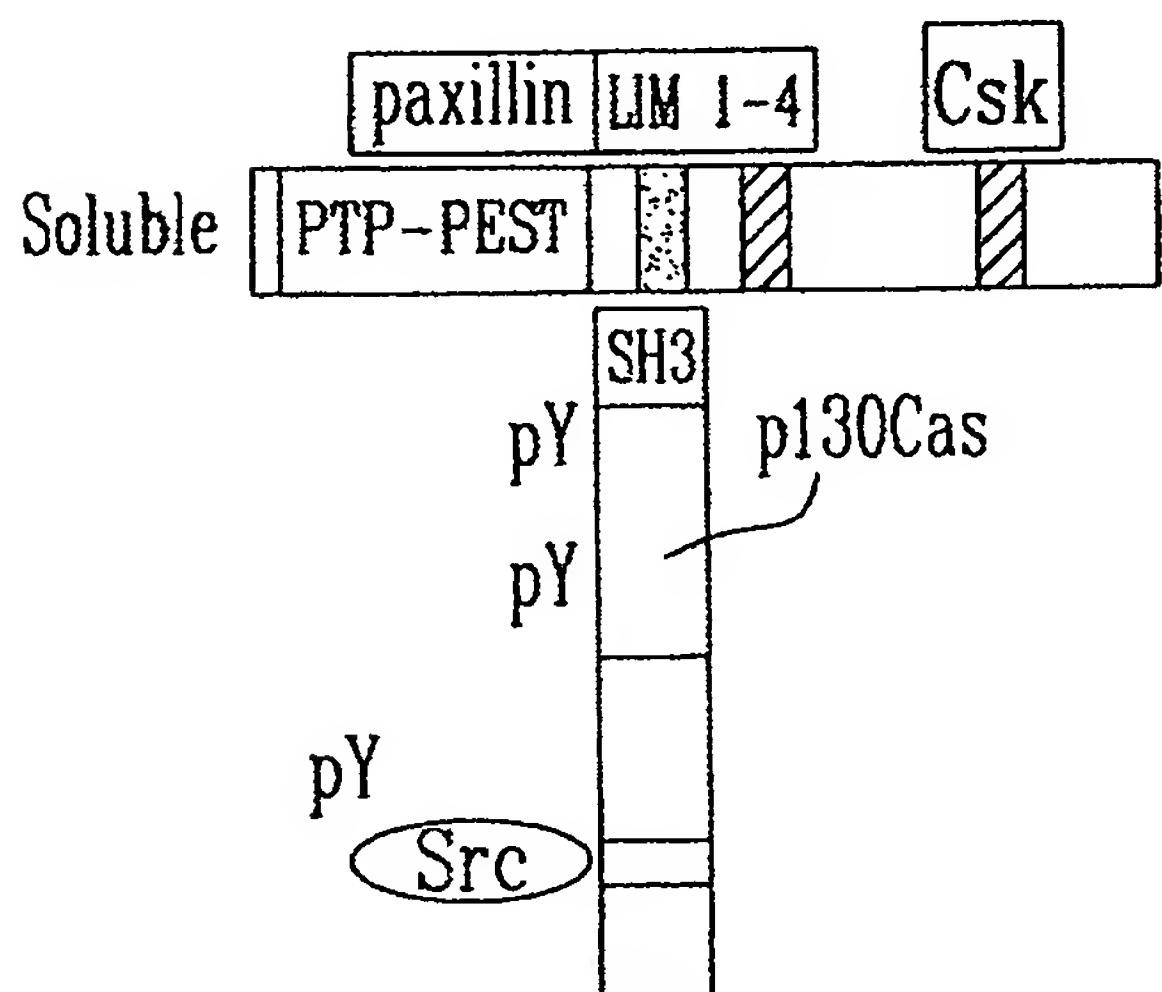
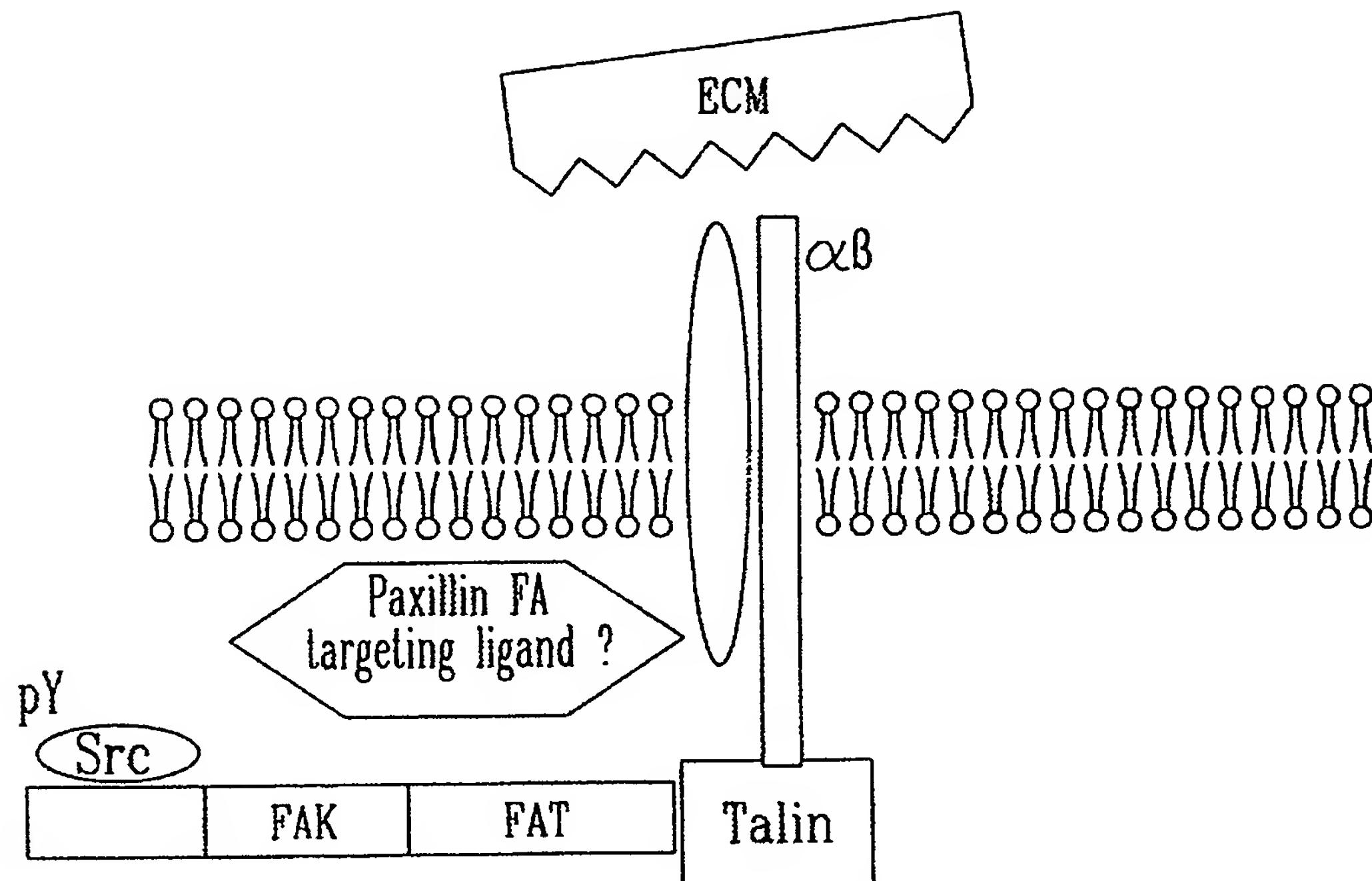


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FIGURE - 23B

MURINE VERSUS HUMAN PEST

MEQVEILRRFIIQRVQAMKSPDHN
+
MEQVEILRKFIQRVQAMKSPDHN

KNRYKDILLPFDHSRVKLTLKTPSQDS
+
KNRYKDILLPFDHSRVKLTLKTPSQDS

IWEYNVVIIVMACREFEMGRKKCERYW
+
IWEYNVVIIVMACREFEMGRKKCERYW

EFQNESRRLYQFHYNVNWPDHDPSSF
+
EFQNESRRLYQFHYNVNWPDHDPSSF

ICAIIDYTWNLLKAGKIPPEEFNVFN
+
ICAIIDYTWNLLKAGKIPPEEFNVFN

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~~75~~ - 24A

1

2

[REDACTED] YEIHQAQKIAADG-NEITTGTMVSSIDSEKQDSPPPKPPRTSCLVEGDAKEEILQPPPEPH 359
 + +
 [REDACTED] YEIHQAQKIAADGVNEINTENMISSIEPEKQDSPPPKPPRTSCLVEGDAKEEILQPPPEPH 360

[REDACTED] PVPPPILTPSPPSAFPTVTTWQDSDRYHPKPVLHMASPEQHPADLNRSYDKSADQWGKSE 419
 +
 [REDACTED] PVPPPILTPSPPSAFPTVTTWQDNDRYHPKPVLHMSSEQHSADLNRNYSKSTELPGKNE 420

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>

SAIEHIDKKLERNLSFEIKKVPLQEGPKSFDGNTILLNRGHAIIKIKSASSSVVDRTSKPQE 479
 + +
 STIEQIDKKLERNLSFEIKKVPLQEGPKSFDGNTILLNRGHAIIKIKSASPCIADKISKPQE 480

3

[REDACTED] LSAGALKVDDVSQNSCADCSAAHSHRAAESSEESQSNSHTPPRDPDCLPLIDKKGHVTWSLH 539
 +
 ++ + +
 [REDACTED] LSSD-LNVGDTSQNSCVDCSVTQSNKVSVTPEESQNSDTPPRPDRLPIIDEKGHVTWSFH 539

GOPENATPVVPDSDGKSPDNHSQTLKTVSSTPNSTAEEAHDLTEHHNSSPLLKAPLSFTN 599
 + + + + + +
 GOPENAIPIPDLSEGNNSSDINYQTTRKTVSLTPSPTTQVETPDLDHDNTSPLFRTPLSFTN 599

~~FBI - 24B~~

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PLHSDDWHDGGSSDGA VTRMKT SISTASATVSPASSAESACHRRVLPMSIARQEVA GTP 659
+ +
PLHSDDSDSDERNSDGA VTQNKT NISTASATVSAATSTESTISTRKVLPMSIARHNIAGTT 659
+ +
HSGAEKDAADVSEESPPPLPERPESFVLA---DMPVURPEWHELPNQEWSEQRESEG LTT S 716
+ +
HSGAEKDVEDVS EDSPPPLPERPESFVLA SERNT PVRSEW SELQS QER SEQKKSEG LITS 719 39/39
+ +
GNEK--HDAGGIHTEASADSPPAFSDKK DQITKSPAEVTDIGFGNRCGKPKGPREP PSEW 774
+ +
ENEKCDHPAGGIHYEMCIECPPTFS DKREQISENPTEATDIGFGNRCGKPKGP RDPPSEW 779
T 775
T 780

~~FIG~~ -24C

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 9/16, C07K 7/06, 14/47, C12N 15/00, A61K 38/00		A3	(11) International Publication Number: WO 99/61467 (43) International Publication Date: 2 December 1999 (02.12.99)
<p>(21) International Application Number: PCT/CA99/00461</p> <p>(22) International Filing Date: 21 May 1999 (21.05.99)</p> <p>(30) Priority Data: 2,238,654 21 May 1998 (21.05.98) CA 60/111,993 11 December 1998 (11.12.98) US </p> <p>(71) Applicant (<i>for all designated States except US</i>): McGILL UNIVERSITY [CA/CA]; 3550 University Street, Montréal, Québec H3A 2A7 (CA).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (<i>for US only</i>): TREMBLAY, Michel, L. [CA/CA]; 420 Touzin, Dorval, Québec H9S 2N2 (CA). CÔTÉ, Jean-François [CA/CA]; 575 Guy #7, Montréal, Québec H3J 2V5 (CA). ANGERS-LOUSTEAU, Alexandre [CA/CA]; 7217 2ème Avenue, Montréal, Québec H2A 3G8 (CA). CHAREST, Alain [CA/US]; 19 Admiral's Way, Chelsea, MA 02150 (US).</p> <p>(74) Agents: DUBUC, Jean, H. et al.; Goudreau Gage Dubuc & Martineau Walker, The Stock Exchange Tower, Suite 3400, 800 Place-Victoria, P.O. Box 242, Montréal, Québec H4Z 1E9 (CA).</p>		<p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> <p>(88) Date of publication of the international search report: 18 May 2000 (18.05.00)</p>	
<p>(54) Title: INHIBITION OF THE BINDING OF PROTEIN TYROSINE PHOSPHATASE PEST TO DOMAINS OF SIGNALLING PROTEINS</p>			
<p>(57) Abstract</p> <p>This invention relates to agents or compounds capable of interfering with the binding of protein tyrosine phosphatase PEST to protein domains of signalling molecules involved in cell migration, focal adhesion and/or cell proliferation, namely p130cas and paxillin. The agents can be derived from the minimal sequences found in binding studies. PTP-PEST is a conserved phosphatase essential for embryo development. Knock-out cells (PTP-PEST -/-) have been perpetuated from null embryos and they show defects in cell migration, considered to be a potential therapeutic agent to treat any disease having any of the following etiological components: cell proliferation, cancer, metastasis, inflammation, and angiogenesis. This invention further relates to a method for finding genuine substrates for enzymes, namely phosphatases, combining gene targeting knock-out technique and substrate-trapping technique with the aid of a substrate binding inactive mutant enzyme. By using a gene targeting knock-out technique, there are less artefacts than by using other techniques (using vanadate compounds, for example) wherein an artificial non-specific increase of the level of hyperphosphorylation occurs.</p>			

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 99/00461

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12H9/16 C07K7/06 C07K14/47 C12N15/00 A61K38/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98 04712 A (COLD SPRING HARBOR LAB) 5 February 1998 (1998-02-05) the whole document	1-8, 10-16, 18-23
Y	WO 95 24419 A (ARIAD PHARMA INC ; RICKLES RICHARD J (US); BRUGGE JOAN S (US); BOTF) 14 September 1995 (1995-09-14) the whole document	1-8, 10-16, 18-23
A	WO 98 00552 A (CHIANG MING KO ; FLANAGAN JOHN G (US); HARVARD COLLEGE (US)) 8 January 1998 (1998-01-08) -/-	1-16

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Patent family members are listed in annex.

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Date of the actual completion of the international search

17 March 2000

Date of mailing of the international search report

24/03/2000

Name and mailing address of the ISA

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Panzica, G

INTERNATIONAL SEARCH REPORT

Int'l. Appl. No.

PCT/CA 99/00461

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GARTON A J ET AL: "IDENTIFICATION OF 130CAS AS A SUBSTRATE FOR THE CYTOSOLIC PROTEIN TYROSINE PHOSPHATASE PTP-PEST" MOLECULAR AND CELLULAR BIOLOGY, US, WASHINGTON, DC, vol. 16, no. 11, 1996, page 6408-6418 XP002051430 ISSN: 0270-7306 cited in the application the whole document	1,2
Y	GARTON A.J. ET AL.: "Association of PTP-PEST with SH3 domain of p130cas: a novel mechanism of protein tyrosine phosphatase substrate recognition" ONCOGENE, vol. 15, no. 8, 1997, pages 877-885, XP000884055 cited in the application abstract	1,2,4,5, 8,12-16
Y	DAVIDSON D. ET AL.: "Inhibitory tyrosine protein kinase p50csk is associated with protein-tyrosine phosphatase PTP-PEST in hemopoietic and non-hemopoietic cells" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 37, 1997, pages 23455-23462, XP000876942 MD US cited in the application abstract figure 1 page 23458, column 2, paragraph 2	1,4,5, 12-16
Y	DOWBENKO D. ET AL.: "Identification of a novel polyproline recognition site in the cytoskeletal associated protein, proline serine threonine phosphatase interacting protein" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 2, 9 January 1998 (1998-01-09), pages 989-996, XP000876966 the whole document	12-16, 18-23
A	SHEN Y. ET AL.: "Direct association of protein-tyrosine phosphatase PTP-PEST with paxillin" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 11, 13 March 1998 (1998-03-13), pages 6474-6481, XP000876943 cited in the application the whole document	1-3,6,7

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 99/00461

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	<p>ANGERS-LOUSTAU A. ET AL.: "Protein tyrosine phosphatase PEST regulates focal adhesion disassembly, migration and cytokinesis in fibroblasts" <i>JURNAL OF CELL BIOLOGY</i>, vol. 144, no. 5, March 1999 (1999-03), pages 1019-131, XP000876950 cited in the application the whole document</p> <p>—</p> <p>CÔTÉ J.F. ET AL: "Intact LIM 3 and LIM 4 domains of paxillin are required for the association to a novel polyproline region (Pro 2) of protein-tyrosine phosphatase PEST" <i>JOURNAL OF BIOLOGICAL CHEMISTRY</i>, vol. 274, no. 29, 16 July 1999 (1999-07-16), pages 10550-20560, XP000876944 the whole document</p> <p>—</p> <p>CÔTÉ J.F. ET AL.: "Combination of gene targeting and substrate trapping to identify substrates of protein tyrosine phosphatases using PTP-PEST as a model" <i>BIOCHEMISTRY</i>, vol. 37, no. 38, 22 September 1998 (1998-09-22), pages 13128-13127, XP000876953 EASTON, PA US cited in the application the whole document</p> <p>—</p> <p>CHAREST A. ET AL.: "Phosphotyrosine-independent binding of SHC to the NPLH sequence of murine protein-tyrosine phosphatase PEST. Evidence for exentended phosphotyrosine binding/phosphotyrosine interaction domain recognition specificity" <i>JOURNAL OF BIOLOGICAL CHEMISTRY</i>, vol. 271, no. 14, 1996, pages 8424-8429, XP000876941 cited in the application the whole document</p> <p>—</p> <p style="text-align: center;">-/-</p>	1,2,4,8, 10, 12-16, 18-23
T		1-7, 12-16, 18-23
Y,P		1,2,6,7, 12-16, 18-23
A		1-7, 12-16, 18-23

INTERNATIONAL SEARCH REPORT

Int'l Application No.

PCT/CA 99/00461

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GARTON A J ET AL: "PTP-PEST: A PROTEIN TYROSINE PHOSPHATASE REGULATED BY SERINE PHOSPHORYLATION" EMBO JOURNAL, GB, OXFORD UNIVERSITY PRESS, SURREY, vol. 13, no. 16, page 3763-3771 XP002051427 ISSN: 0261-4189 abstract page 3763, paragraph 1	1,10,11
A	CHAREST A ET AL: "MURINE PROTEIN TYROSINE PHOSPHATASE-PEST, A STABLE CYTOSOLIC PROTEIN TYROSINE PHOSPHATASE" BIOCHEMICAL JOURNAL, GB, PORTLAND PRESS, LONDON, vol. 308, no. PART 02, 1995, page 425-432 XP000676759 ISSN: 0264-6021 cited in the application the whole document especially Fig.1	1,4-7
Y	LIU F ET AL: "DIRECT BINDING OF THE PROLINE-RICH REGION OF PROTEIN TYROSINE PHOSPHATASE 1B TO THE SRC HOMOLOGY 3 DOMAIN OF P130CAS" JOURNAL OF BIOLOGICAL CHEMISTRY, US, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 271, no. 49, 1996, page 31290-31295 XP002051433 ISSN: 0021-9258 cited in the application the whole document especially abstract, figures 1 and 2	1,2,4-7, 12-16, 18-23
Y	YANG Q ET AL: "CLONING AND EXPRESSION OF PTP-PEST A NOVEL, HUMAN, NONTRANSMEMBRANE PROTEIN TYROSINE PHOSPHATASE" JOURNAL OF BIOLOGICAL CHEMISTRY, US, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 268, no. 9, 1993, page 6622-6628 XP002034265 ISSN: 0021-9258 the whole document especially abstract and fig. 1	1,4-7
		-/-

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 99/00461

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FAUMAN E B ET AL: "Structure and function of the protein tyrosine phosphatases" TIBS TRENDS IN BIOCHEMICAL SCIENCES, EN, ELSEVIER PUBLICATION, CAMBRIDGE, vol. 21, no. 11, 1996, page 413-417 XP004071016 ISSN: 0968-0004 _____	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA 99/00461

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims No.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims No.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. Claims No.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims No.:

4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claim No.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 12-15 relate to an extremely large number of possible methods. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the methods claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the methods as claimed in cl. 12-15 where the enzyme mentioned therein is meant to be PTP-PEST (as in cl. 16).

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int'l. Appl. No.

PCT/CA 99/00461

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